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RESEARCH ARTICLE

Persistence of Antibacterial Resistance and Virulence Gene Profile of Methicillin Resistant Staphylococcus Aureus (MRSA) Isolated From Humans and Animals

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ABSTRACT

The persistence of antibacterial resistance and virulence gene profile of well characterized MRSA isolated from animals and human was determined using antibiotic susceptibility testing and PCR amplification of virulence and methicillin resistance gene. Antibiotic susceptibility testing revealed a general reduction in the rate of resistance to antibiotics previously tested. Isolates were currently susceptible to minocycline a tetracycline derivative, amikacin and gentamicin respectively. Resistance to cefoxitin and oxacillin were currently observed in 64 and 79% of all the isolates which in the case of cefoxitin it was less than the 86% while a bit higher in oxacillin as reported in the previous study. In addition, currently 57%, 43% and 36% of the isolates were resistant to amoxicillin, tetracycline and erythromycin which is less than the isolates previous resistance profile to amoxicillin and erythromycin whereas unchanged in the case of tetracycline. Four (29%) of the isolates were also currently resistant to vancomycin, doxycycline and amoxicillin-clauvulanic acid; while only two isolates were resistant to vancomycin and three isolates were resistant to doxycycline in the previous study. No changes were observed in the number of isolates resistant to amoxicillin-clauvulanic acid. Resistance to more than one class of antibiotics was observed in 64% of the isolates. Currently we observe loss of methicillin resistance determinants *mec*A and susceptibility to all antibiotics tested in three isolates and reduced susceptibility in two isolates.

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INTRODUCTION

The persistence and spread of multi drug resistant and virulent strains of methicillin resistant *S. aureus* (MRSA) is a major global public health problem (Tenover, 2006). The significance of methicillin resistant *S. aureus* to public health is the ability of the pathogen to rapidly acquire resistance and virulence gene thus, paving way for the emergence of new and highly pathogenic clones making treatment with antibiotics difficult and prolonging hospital admission stay (Liu, 2009).

The emergence of several MRSA clones coupled with the organism's rapidly changing epidemiology on a major scale has a profound clinical implication on the patient, animal husbandry, veterinary and health care workers since, new clones harbors different resistance and virulence determinants (Chua *et al*., 2014). In addition, persistence of resistance development due to horizontal transfer of resistance determinants, selective antibiotics pressure, nasal colonization, small colony variant and clone replacement have rendered the therapeutic value of antibiotics ineffective (Chua *et al*., 2014). Further studies have also revealed that MRSA are evolving to be more competent in the face of environmental stress, and are also known producers of a number of potent virulence factors involved in disease process (Gould *et al*., 2012; Lim *et al*., 2012).

The threat posed by resistance to antibiotics is growing at an alarming rate, possibly even more rapid in countries where there is poor legislation with regards to prescription and use of antibiotics. Apart from indiscriminate use of antibiotics, a number of factors play a role in the rapid growth and spread, ranging from the natural traits inherent in bacteria which it deploys to face

the therapeutic effect of antibiotics, to regulatory and economic issues behind antibiotic abuse. Understanding the factors influencing the persistence and spread of MRSA will help in providing the impetus towards designing an efficient strategy vital to the prevention and control of MRSA infection. We hypothesized that antibiotic resistance gene persist and that MRSA harbors a number of putative virulence factors. This study was designed to determine the persistence of antibiotic resistance and virulence gene profiles of well characterized MRSA isolated from animals and humans in Malaysia.

MATERIALS AND METHODS

Bacterial Isolates: A total of 14 MRSA stock cultures obtained from animals (3 dogs, 1 cat, 2 chicken), 1 environmental sample and 7 humans isolates from previous studies (Abdulkadir, 2012; Erkihun, 2010) were used in this experiment. The isolates were collected from 2008-2011 and were identified as *S. aureus* using biochemical test and Staphylococcus identification kit, Staphytect plus(R) (Oxoid, UK) and Dry spot™ Staphytect plus (DR0100M, UK) respectively according to the manufacturers recommendation before they were stored in cryobeads tubes at -80□C. The cultures were revived by sub-culturing onto blood agar and were reconfirmed as *S. aureus* by biochemical tests and PCR amplification of the endonuclease species specific *nuc* gene. MRSA confirmation was carried out by culturing onto oxacillin screening agar base (ORSAB) (Oxoid, UK) and PCR amplification of methicillin resistance determinants *mec*A.

Antibiotic susceptibility test: Antibiotic susceptibility testing was performed using Kirby-Bauer disk diffusion. The inhibition zone diameter was measured and interpreted according to the guidelines of Clinical Laboratory Standard Institute (CLSI, 2014). All isolates were tested for susceptibility to ten critically important antibiotics amoxicillin 25µg, minocycline 30µg, imipenem 10µg, amikacin 30µg, amoxicillin-clauvulanic acid 30µg, doxycycline 30µg, tetracycline 10µg, erythromycin 15µg, streptomycin 10µg, vancomycin 30µg, and three highly important antibiotics, i.e., oxacillin 1µg, cefoxitin 30µg, gentamicin 10µg, and (Oxoid, UK).

Polymerase chain reaction: Genomic DNA was extracted from overnight grown cultures by boiling method as previously described by (Chen *et al*., 2009) but without the use of lysostaphin. The detection of methicillin resistance determinant *mec*A and ten selected virulence genes were performed in a 50µL reaction volume. The reaction was performed in a thermocycler (Bio-Rad) using specific primers (IDT Singapore) and appropriate PCR cycling conditions as previously described by Mehrotra *et al*. (2000) for *mec*A, *et*A and B, Sapri *et al*. (2011) for *Pvl* and *tst-1*, Williams *et al*. (2000) for *set-1*, Zdzalik *et al*. (2012) for *Ssp*A, Kumar *et al*. (2011) for *hlα* and *hlβ*, Chiang *et al*. (2008) for *Seu* and Said-Salim *et al*. (2003) for *geh* (Table 1). Electrophoresis of PCR products was carried out in 2% agarose (Sigma-Aldrich) prepared in a 0.5X TBE buffer. Ten microliter (10 µL) of PCR products each were electrophoresed at 80V for 90min and then visualized under the transilluminator UV-light using a gel documentation system alpha imager (Bio-Rad).

RESULTS

Antibiotic susceptibility test: Persistence of antibiotic resistance of MRSA isolates were determined by comparing their current resistance profile with the isolate's previous resistance profile as shown in Table 2. Antibiotic susceptibility testing revealed a general reduction in the rate of resistance to antibiotics previously tested. Currently all isolates were susceptible to minocycline a tetracycline derivative, amikacin and gentamicin respectively while not all isolates were resistant to the same antibiotics as observed in the previous study. Resistance to cefoxitin and oxacillin were currently observed in 64 and 79% of all the isolates which in the case of cefoxitin it was less than the 86% as reported in the previous study. However, in the case of oxacillin, the resistance profile was higher than the 57% as observed in the previous study. We currently observed 57%, 43% and 36% resistance to amoxicillin, tetracycline and erythromycin which is a much lower resistance profile as compared to the isolates' previous resistance profile to both amoxicillin and erythromycin. There was no change in the number of isolates resistant to tetracycline in both studies. Four (29%) of the isolates were currently observed to be resistant to vancomycin, doxycycline and amoxicillin-clauvulanic acid; however, when compared with their previous resistance profile we noticed that only two isolates were resistant to vancomycin while only three isolates were resistant to doxycycline. There were no changes in the number of resistance to amoxicillin-clauvulanic acid both in the current and previous studies. Resistance to more than one class of antibiotics was observed in 64% of the isolates. Amplification of methicillin resistance determinant revealed that three isolates were currently *mec*A negative and were also susceptible to all antibiotics tested which were not the case as observed in the previous study.

PCR amplification of virulence gene: Results of PCR amplification of genes coding for virulence determinants are shown in Figure 1 and 2 which revealed that 4 (29%) of the isolates were positive for the phage-borne Panton valentine leucocidin (*Pvl*), 57% for beta hemolysin (*hlβ*), 86% for Staphylococcus exotoxin-like toxin 1(*set1*). In addition, 21% were positive for v8 protease (*SspA*) and exfoliative B (*etB*) respectively. Similarly, 7.1% were positive for *tst*, exfoliative A (*etA*) and alpha hemolysin (*hlα*). In addition, 14.3% were positive for gene coding for staphylococcus enterotoxin u (*Seu*) and lipase encoding gene (*geh*) as depicted in Table 3.

DISCUSSION

The study on persistence of antibacterial resistance and virulence gene profile was designed in order to determine the potentials of MRSA in retaining their resistance capabilities even after prolonged storage at

Table 1: Nucleotide sequences and anticipated sizes of PCR products for the *S. aureus* gene-specific oligonucleotide primers used in this study

Gene	Primer set	Oligonucleotide sequence	PCR conditions		Product size(bp)	References
			Temp (°C)	Time (s)		
Nuc	NUC-F	5'-GCG ATT GAT GGT GAT ACG GTT-3'	94	60	278	Saiful *et al.* (2006)
	NUC-R	5'-AGC CAA GCC TTG ACG AAC TAA AGC-3'	55	30		
			72	120		
mecA	MECA-F	5'-ACT GCT ATC CAC CCT CAA AC-3'	94	120	163	Mehrotra *et al.* (2000)
	MECA-R	5'-CTG GTG AAG TTG TAA TCT GG-3'	57	120		
			72	60		
Tst-1	TST-F	5'-TGA TAT GTG GAT CCG TCA T-3'	94	120	387	Sapri *et al.* (2011)
	TST-R	5'-AAA CAC AGA TGG CAG CAT-3'	58	60		
			72	120		
Seu	SEU-F	5'-ATT TGC TTT TAT CTT CAT-3'	94	30	167	Chiang *et al.* (2008)
	SEU-R	5'-GGA CTT TAA TGT TTG TTT CTG AT-3'	51	30		
			72	30		
Set-1	SET-1-F	5'-GGG ACA GAA TAA TAC TAT GAA ATT AA	94	60	253	Williams *et al.* (2000)
	SET-1-R	AAA CG-3'	53	60		
		5'-ATC TTT TTG GTT AAA GCG TAC-3'	72	60		
Pvl	PVL-F	5'-ATG TCT GGA CAT GAT CCA A-3'	94	120	970	Sapri *et al.* (2011)
	PVL-R	5'-AAC TAT CTC TGC CAT ATG GT-3'	58	60		
			72	120		
sspA	SSPA-F	5'-GCG ACA CTT GTG AGT TCT CCA GC-3'	95	60	772	Zdzalik *et al.* (2012)
	SSPA-R	5'-GTT TTA AGA AGT TGC GTA CAT TTT C-3'	50	60		
			72	60		
Geh	GEH-F	5'-GCA CAA GCC TCG G-3'	94	60	319	Said-Salim *et al.* (2003)
	GEH-R	5'-GAC GGG GGT GTA G-3'	50	60		
			72	60		
etA	ETA-F	5'-GCA GGT GTT GAT TTA GCA TT-3'	94	120	93	Mehrotra *et al.* (2000)
	ETA-R	5'-AGA TGT CCC TAT TTT TGC TG-3'	57	120		
			72	60		
etB	ETB-F	5'-ACA AGC AAA AGA ATA CAG CG-3'	94	120	226	Mehrotra *et al.* (2000)
	ETB-R	5'-GTT TTT GGC TGC TTC TCT TG-3'	57	120		
			72	120		
Hlα	HLα-F	5'-GGT TTA AGC CTG GCC TTC-3'	94	30	543	Kumar *et al.* (2011)
	HLα-R	5'-CAT CAC GAA CTC GTT CG-3'	50	30		
			72	60		
Hlβ	HLβ-F	5'-GCC AAA GCC GAA TCT AAG-3'	94	30	833	Kumar *et al.* (2011)
	HLβ-R	5'-GCG ATA TAC ATC CCA TGG C -3'	50	30		
			72	60		

Table 2: Comparison of Antibiotic Susceptibility and Virulence Gene Profile of MRSA Isolated From Humans and Animals

ID	Resistance Profile (I)	Resistance Profile (II)	Virulence gene
SH1	AK, AML, OX, FOX, S, MH, AMC, CN, IPM, TE, E(11)	AML, OX, FOX, S, VA, TE, E(7)	*mecA, etB, geh, hlb, Set1, pvl*
SH2	AK,AML,S,CN(4)	-(0)	*etA, hlb, Set1,Seu*
SH3	AK,AML,OX,FOX,S,MH,CN,TE,E(9)	AML, OX, FOX, AMC, E(5)	*mecA, Set1, Seu, tst*
SH4	AK,S,MH,TE(4)	AML, FOX, OX, S, AMC, DO, TE, E(8)	*mecA, etB, hlb, Set, pvl*
SH5	AK, AML, OX, FOX, S, MH, DO, AMC, CN, TE, E(11)	OX(1)	*hlb, Set1*
SH6	AML, OX, MH, AMC(4)	OX, FOX(2)	*SspA*
SH7	AK, OX, S, MH, CN, VA(6)	-(0)	*etB, Set1*
SCT1	AK, AML, OX, FOX(4)	AML, OX, FOX(3)	*mecA, hla, Pvl*
SEV1	AK, AML, OX, FOX, MH, DO, AMC, CN, TE, E, VA(11)	AML, OX, FOX, S, AMC, TE, E,VA(8)	*mecA, hlb, set1*
SDG1	AK, AML, OX, FOX(4)	-(0)	*Set1*
SDG2	AK, AML, OX, FOX, S, AMC, E(7)	OX, FOX, S, DO, TE, E,VA(7)	*mecA, hlb, Set1, SspA*
SDG3	AML, OX, FOX, S(4)	AML, OX, FOX, S, AMC, DO, TE, E, VA(9)	*mecA, hlb, Set1,pvl*
SCH1	AK, AML, OX, FOX, S, DO, TE, E(8)	OX(1)	*Set1*
SCH2	AML, OX, MH(3)	OX, FOX, S, TE (4)	*mecA, hlb, Set1*

(II): current profile

Table 3: Virulence gene profiles of local MRSA isolates

Source	ID	Virulence gene											
		nuc 267bp	*mec*A 163bp	*et*A 93bp	*et*B 226bp	*geh* 319bp	*hlβ* 543bp	*Hlα* 833bp	*Set*1 253bp	*Seu* 167bp	*Ssp*A 772bp	*tst* 387bp	*Pvl* 970bp
Humans	SH1	+	+	-	+	+	+	-	+	-	-	-	+
	SH2	+	-	+	-	+	+	-	+	+	+	-	-
	SH3	+	+	-	-	-	-	-	+	+	-	+	-
	SH4	+	+	-	+	-	+	-	+	-	-	-	+
	SH5	+	-	-	-	-	+	-	+	-	-	-	-
	SH6	+	-	-	-	-	-	-	-	-	+	-	-
	SH7	+	-	-	+	-	-	-	+	-	-	-	-
Dog	SDG1	+	-	-	-	-	-	-	+	-	-	-	-
	SDG2	+	+	-	-	-	+	-	+	-	+	-	-
	SDG3	+	+	-	-	-	+	-	+	-	-	-	+
Environment	SEV1	+	+	-	-	-	+	-	+	-	-	-	-
Cat	SCT1	+	+	-	-	-	-	+	-	-	-	-	+
Chicken	SCH1	+	-	-	-	-	-	-	+	-	-	-	-
	SCH2	+	+	-	-	-	+	-	+	-	-	-	-
	TOTAL	100%	57%	7%	21%	14%	57%	7%	86%	14%	21%	7%	29%

+: positive , - : negative

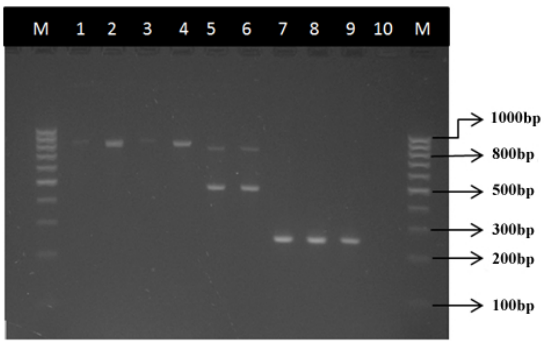

Fig. 1: PCR amplification of virulence gene of MRSA isolated from humans and animals, Lane 1-4 showing hlβ 833bp gene, lane 5 & 6 showing SspA 772bp & geh 319, lane 7-9 set1 253bp, lane10 negative control resolved in 2% agarose gel electrophoresis using 0.5X TBE buffer at 80V for 90min. (M= 100bp gene Exact DNA maker, Fisher Scientific).

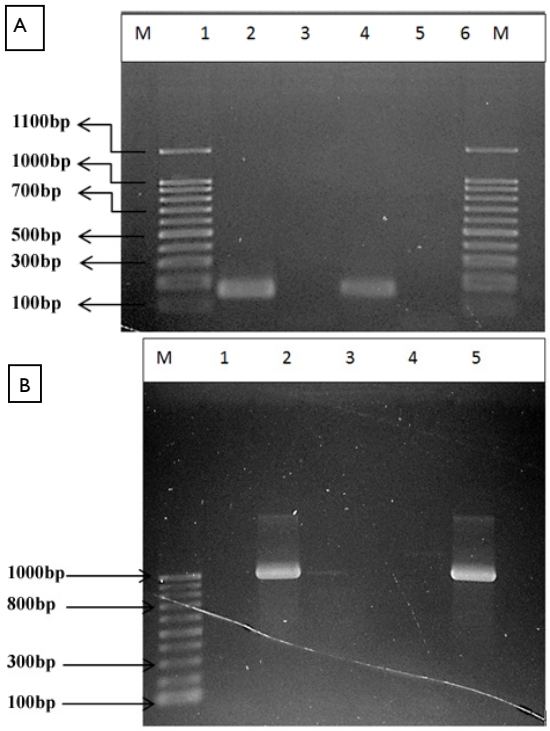

Fig. 2 (a): PCR amplification of mecA gene in MRSA lane 1 mecA 163bp, lane 2 *Staphylococcus aureus* ATCC25923, lane 3 positive control, lane 4 negative control**, (b):** lane 1 negative control, lane 2&6 PvI 970bp resolved in 2% agarose gel electrophoresis using 0.5X TBE buffer at 80V for 90min.

-80ºC with a view to regulating the spread of antibacterial resistance. In this study, a general increase was observed in susceptibility to antibiotics amongst MRSA isolates after prolonged storage at -80ºC. Initial resistance profile of the isolates revealed a significant decrease in resistance to amikacin, amoxicillin and oxacillin, as compared to their current profile.

Similar findings were also observed in resistance to doxycycline and streptomycin however, no variation was observed in the isolates' resistance to amoxicillin-clauvulanic acid, imipenem, tetracycline and erythromycin. Currently none of the isolates were resistant to amikacin, gentamicin and minocycline, however, intermediate resistance was observed. This variation observed in the pattern of resistance may have been contributed by lack of prior exposure of the isolates to antibiotics, duration and condition of storage (Van Griethuysen *et al*., 2005) or perhaps loss due to mutation. Resistance to vancomycin observed might be due to emergence of low–level vancomycin resistance strains (Howden *et al*., 2006), this finding have clinical implication, since glycopeptides are considered drug of last resort in the treatment of *S. aureus* infection and emergence of low level resistance strains have been reported as the cause of treatment failures associated with MRSA infections (Song *et al*., 2004). Currently, reduced susceptibility to antibiotics tested was observed in two isolates SDG3 (dog) and SH4 (human) as compared to their previous resistance profile which shows they were susceptible to quiet a number of antibiotics tested. This unique change in resistance profile observed in the isolates could be due to the effect of incubation temperature and content of the culture medium as well as the duration of incubation (Rohrer *et al.*, 2003).

The presence of *mec*A gene is seen as a benchmark in determining methicillin resistance in *S. aureus*. The loss of *mec*A gene in three isolates may possibly be due to the effect of prolonged storage; mutation or loss due to fitness cost, another explanation could be that since MRSA exhibit hetero-resistance, it is not however impossible to assert that the cryobeads taken from the stock cultures at the point of inoculation is embedded with a small colony variant of *mec*A negative strain of MRSA. Nonetheless, this finding is consistent with the report by Van Griethuysen *et al*. (2005) who observed a loss of *mec*A in 14.4% out of the 250 MRSA cultures after prolonged storage at -80℃. similar findings were reported by other researchers (Hürlimann-Dalel *et al*., 1992; Berger-Bächi and Tschierske, 1998; Katayama *et al*., 2000). In addition, hyper production of altered penicillin binding proteins and other unidentifiable factors are responsible for increase resistance to methicillin in some *mec*A negative S. *aureus* (Chambers, 1997) and this could also result in the isolation of *mec*A negative strain even though phenotypically it was resistant to methicillin and cefoxitin. Such unique features may lead to the suspicion that those *mec*A deficient strains might be those MRSA showing borderline resistance towards oxacillin, borderline oxacillin resistant *S. aureus* are hyper producers of altered penicillin binding protein or penicillinase (Brown, 2001). Several other factors are known to have played a role in the development of methicillin resistant in *S. aureus,* absence of *mec*A can be associated with a range of factors, since there are other mechanism that contribute to the development of methicillin resistance in *S. aureus* (Berger-Bachi and Tschierske, 1998). Expression of methicillin resistance has been shown to be influenced by the genes involved in precursor turnover, transport and regulation, and finally signal transduction (Berger-Bächi and Rohrer, 2002); which could lead to failure of detection methicillin resistance in *S. aureus.*

PCR amplification of virulence gene revealed that the carriage rate of virulence determinants were relatively higher in human than our animals' isolates. However, with majority of our isolates positive for the commonly

isolated food poisoning-borne staphylococcal exotoxin- 1 (*set1*), Staphylococcus exotoxin like toxin (*set*1) is an exotoxins which are involve in staphylococcal food poisoning, stimulation of T-lymphocytes and toxic shock syndrome. They share similar biological activities with streptococcal exotoxins and hence the name pyrogenic toxins (Williams *et al*., 2000). Furthermore, studies have revealed that 40% of clinical *S. aureus* are known to produce at least one of the four exotoxin types (Schmitz *et al*., 1998). This finding is in congruent with our studies since about 86% of our isolates were positive for the gene coding for virulence even though the sample size was low. In addition, the high carriage rate of *Set*1 in our isolates could be due to the fact that since majority of *S. aureus* superantigens are located on the mobile genetic elements and thus can be easily acquired horizontally from other cells.

We also observed that 7% and 57% of the isolates respectively were positive for the gene coding for alpha and beta hemolysin. Hemolysin are pore-forming water soluble toxin known to be expressed by 95% *S. aureus* isolates and possesses strong affinity for epithelial cells, macrophages, monocytes, fibroblast and erythrocytes and known to be regulated by accessory gene regulator (Grumann *et al*., 2014). Both hlα and hlβ have affinity for rabbit and sheep erythrocyte respectively and are very important in screening for *S. aureus* on blood agar. In addition, hlα is mostly seen in animal isolates which was consistent with our finding as only one isolate from cat was positive for α-hemolysin. Furthermore, four of our isolates were positive for the community associated methicillin resistant *S. aureus* (CA-MRSA) phage-borne associated Panton Valentine leucocidin toxin (PVL). PVL is an apparent virulence factor that is known to play a significant role in the pathogenesis of community acquired MRSA infection with high ability to stimulate cytolysis of the neutrophils, apoptosis and elaborating pro-inflammatory molecules (Liu, 2009). This explains the severity of CA-MRSA infections seen in humans since it targets inflammatory cells. PVL toxins are also responsible for skin and soft tissue infection in humans and commonly seen in USA 300 and 400 CA-MRSA strain (Chua *et al*., 2014). This toxin however, was never isolated in HA-MRSA, thus, indicating the possibility that we are dealing with a community acquired MRSA. Other genes found, includes the scalded skin syndrome exfoliative toxin A and B commonly isolated in human isolates. In addition it has been isolated in a case of CA-MRSA infection in Japan (Bukowski *et al*., 2010). This finding is also in congruent with our finding as one of our isolate (SH4) harbors both *etB* and *Pvl* gene. Our isolates were also positive for toxic shock syndrome toxin (*tst)*, Protease (*SspA*) and Lipase (*geh*), the role of the latter two in the pathogenesis of *S. aureus* is poorly understood except that protease are interferes with complement associated bacterial killing. Expression of virulence determinants is controlled by the accessory gene regulators and that could explain the reason why high carriage rate was observed in some isolates while low in others.

Conclusions: Increase in susceptibility to antibiotics was observed in all isolates after prolong storage at -80 °C. In addition, relatively high carriage rate of the virulence determinants in our isolates were also observed. These findings affirms the importance of resistance and virulence determinants to successful adaptation of MRSA and emphasize the need for further research on the mechanism of antibiotic resistance acquisition and the role of virulence as well as regulatory genes in the pathogenesis of MRSA with a view to ensuring effective prevention and control of MRSA spread.

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