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Characterisation of Methicillin-resistant *Staphylococcus aureus* isolated from blood cultures in South East Asian hospitals.

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Characterisation of Methicillin-resistant *Staphylococcus aureus* isolated from blood cultures in South East Asian hospitals.

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ABSTRACT:
Methicillin-resistance Staphylococcus aureus (MRSA) are common in hospitals in many countries, including South East Asia. Nosocomial MRSA is characteristically resistant to multiple antibiotics but can be treated with vancomycin. More recently MRSA have emerged in the community (CMRSA) and these characteristically are not multiply resistant like the hospital strains. MRSA have been found to spread within and between hospitals, thus are referred to as epidermic MRSA (EMRSA). It is therefore important to characterise isolates so that pathogenic and epidemic strains of MRSA can be identified. For this study 309 MRSA isolated from blood cultures between June 1998 and December 1999 were provided by the Sentry Program Centre in Adelaide, South Australia. The isolates were from hospitals in Australia, South Africa, Singapore, China, Hong Kong, Taiwan, the Philippines and Japan. Isolates were characterised by phenotypic and molecular methods. The methods used were extended antibiograms and resistograms, bacteriophage typing, countour-clamped homogeneous electric field (CHEF) electrophoresis, plasmid profiling and analysis of the mec complex. Most of the isolates were resistant to the majority of the antimicrobials tested although all were susceptible to vancomycin. Three isolates from Hong Kong and seven from Royal Perth hospitals had resistance profiles similar to those of CMRSA. The majority of isolates were not susceptible to the International Bacteriophage Set (IBS). There was a predominant CHEF pattern amongst the South African isolates which had 88% similarity with the Australian CHEF pattern 1. However, the isolates were not related in other respects and had different plasmid profiles and mec complexes. The results indicated that the South African isolates are different from those from other hospitals and that Taiwanese and Japanese isolates are generally more diverse than those in the other countries. Hospitals in Australia, Singapore and Hong Kong appear to have many different strains but do not have predominance of a particular strain. This study has provided a basis for additional studies to further characterise isolates from various countries and to understand the epidemiology of MRSA in the hospitals.

Key Words: MRSA, CHEFF, Methicillin, Staphylococcus, Bacteriophage, Plasmids, Typing, Antibiograms, Resistograms, Hospitals
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INTRODUCTION:

Staphylococcus aureus (S.aureus) is a Gram-positive coccus, found in approximately 30% of healthy individuals especially on the skin, nose and upper respiratory tract [1]. It is also found in hospitals, on the clothes, bedding and instruments. It is a pathogen that causes a range of infections from superficial skin infections to severe and life threatening infections. It is a major cause of nosocomial infections [1]. It has been reported that S.aureus is the most frequent cause of bacteraemia in the hospital environment [1]. S. aureus has become increasingly resistant to antibiotics [2, 3]. The antibiotic methicillin was introduced in 1959 to control the strains of S.aureus that had become resistant to penicillin and other antibiotics [4]. Shortly after its introduction, strains of S.aureus were isolated that were resistant to methicillin [5, 6]. These strains were referred to as methicillin-resistant Staphylococcus aureus (MRSA). In South East Asian countries, the isolation rates for MRSA have increased and have caused outbreaks of infections in hospitals [7]. The strains have been referred to as "Southeast Asian MRSA"(SEA-MRSA) because of their origin [8]. MRSA isolated from blood cultures has been reported to be a significant pathogen causing bacteraemia in Asian countries [6, 8]. The high percentage of MRSA reported from bacteraemias in recent years may have occurred because of failure of the immune system to contain the infection at the focal site, prolong use of antibiotics and indwelling devises [9]. The aims of this study were to use conventional methods to type MRSA isolates from cases of bacteraemia in South East Asian hospitals. To use the typing results obtained to determine the particular strains of MRSA that are responsible for bacteraemia within and between hospitals.

MATERIAL AND METHODS:

MRSA isolates: A total of 309 MRSA isolated from blood cultures between June 1998 and December 1999 were provided by the Sentry Program Centre in Adelaide, South Australia. The isolates were from three hospitals in Australia, One in South Africa and eleven hospitals from six Asian countries (Singapore, China, Hong Kong, Taiwan, Philippines and Japan).

Typing methods: Susceptibility tests (Antibiogram and Resistogram): Antibiogram typing was performed on Mueller-Hinton agar (Oxoid) by the single disc diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines [10] against a range of 21 antimicrobial agents (Oxoid): amikacin (Ak-30µg), fusidic acid (Fd-10µg), kanamycin (K -30µg), minocycline (Mh-30µg), mupirocin (Mup-5µg), neomycin (N-

30µg), spectinomycin (Sh-25µg), streptomycin (S-25µg), sulphafurazole (Sf-300µg), lyncomycin (My-15µg), tobramycin (Tob-10µg) and trimethoprim (W-5µg). Resistogram typing was performed on Mueller-Hinton agar (Oxoid) by the single disc diffusion method against a range of 6 chemicals [10]: cadmium acetate (Cd-0.1 M) from Chem-Supply limited Australia, ethidium bromide (Eb-15mM) from Sigma Chemical limited Australia, mercuric chloride (HgCl- 0.4µM), phenylmercuric acetate (Pma-0.17%) from Ajax Chemical limited Australia, propamidine isethionate (Pi -1%) from May and Baker limited United Kingdom and sodium arsenate (Ars-0.2µM) from BDH Chemical limited United Kingdom.

Bacteriophage typing: Bacteriophage typing was performed on 3CY agar by using the appropriate propergating strain of S. aureus against the international basic set (IBS) of 26 phages: Group I - 29, 52, 52A, 79, 80; Group II - 3A, 3C, 55, 71; Group III - 6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85; Group IV - 94, 96; Miscellaneous - 81, 96; Experimental phage - 187,90,88 and the international MRSA set (IMS) of 10 phages: MR8, MR12, MR25, F30, F33, F38, M3, M5, 622, 56B. Both sets were prepared to 100 times routine test dilution (RTD) according to the method of Blair and Williams [11].

Contour-clamp homogeneous electric field electrophoresis (CHEF): CHEF method used was adapted from Wei and Grubb [12] and O'Brien et al., [13] with the CHEF DR III System (Bio-Rad Laboratories Pty Ltd, Regents Park, New South Wales). Chromosomal banding pattern were examined visually, scanned with a Fluor-S Multimager (Bio-Rad Laboratories) and digitally analysed with Multi-Analyst/PC (Bio-Rad Laboratories). The CHEF patterns were grouped according to Tenover et al., [14] and using the dendogram similarities of 80% to assign strain relatedness. S. aureus NCTC 8325 was used as the size standard.

Plasmid profiles: Plasmid typing was performed by isolating the plasmid DNA by the cetyl-trimethyl-ammonium-bromide (CTAB) method [15] and separated by horizontal-gels electrophoresis in 0.6% (wt/vol) molecular biology-grade agarose (Promega Corporation, United State of America). Plasmid DNA pattern were examined visually, scanned with a Fluor-S Multimager (Bio-Rad Laboratories) and digitally analysed with Multi-Analyst/PC (Bio-Rad Laboratories). Plasmid sizing were determined by comparing the migration of the covalently closed circular (CCC) forms with the plasmid of WBG 4483 that had the standard sizes of 44.3 kb, 22.5kb, 4.4kb, and 3.5kb using the QWBASIC Corona PC Basic Version 1.04 Corona Data System to

calculate the sizes. Transfer of plasmids from selected isolates to WBG1876 was attempted using the Mixed-culture transfer (MCT) as described previously [16]. WBG1876 transipients were selected on media containing fusidic acid (5µg/ml) and rifampin (25µg/ml) (Sigma Chemical Company) with a range of antimicrobial agents for selection plates. Transferrants were analysed for plasmids as previously described [15]. Isolates were examined for conjugative plasmids using the polyethylene glycol (PEG) method of Townsend et al., [16] using WBG541 as recipient. Transferred conjugative plasmids were analysed as previously described [15]. Restriction endonucleases EcoRI and HindIII (Promega) analysis of transferred plasmids was digitally analysed according to the manufacturer's directions. The fragments are separated by horizontal-gels electrophoresis in 0.8% (wt/vol) molecular biology-grade agarose (Promega Corporation, United State of America) with Multi-Analyst/PC (Bio-Rad Laboratories). The DNA sizing was determined by comparing the sizes of plasmid digested fragments with those of EcoRI/HindIII digested Lambda DNA that has fragments of 21,227 bp, 5148 bp, 4268 bp, 3530 bp, 1904 bp, 1584 bp, 1375 bp, 947 bp, 831 bp and 564 bp using the QWBASIC Corona PC Basic Version 1.04 Corona Data System to calculate the sizes.

Analysis of mecA complex: Chromosomal DNA was prepared to the method of Sambrook et al., [17]. The mecA complex of the isolates was analysed digitally using primers designed to target specific areas of the mec region and the PCR amplification of the mecA gene was performed as described previously [17]. Chromosomal sizing of the amplification products were measured using the QWBASIC Corona PC Basic Version 1.04 Corona Data System to calculate the sizes. Restriction enzymes (EcoRI and HindIII) analyses for amplicons of mecA and mecI2 primers were digested with ClaI in order to detect deletions in the membrane-spanning binding region of mecR1. The sizes of amplication products were measured using the QWBASIC Corona PC Basic Version 1.04 Corona Data System to calculate the size.

RESULTS:

Susceptibility tests (Antibiogram and Resistogram): MRSA isolates were resistant to the majority of the antimicrobial agents and chemicals tested. However, susceptibility to vancomycin alone was found in all isolates from the fifteen hospitals studied. Bacteriophage typing: Variable bacteriophage lysis patterns were found amongst the isolates. However of the common bacteriophage patterns identified (Table 1), only seven bacteriophage

patterns belonging to Group III were found in more than one country. With 80 isolates belonging to the untypable pattern. Contour-clamp homogeneous electric field electrophoresis (CHEF): Fifty CHEF patterns as illustrated in figure 1 were identified by PFGE, although prominent CHEF patterns as shown in Table 2 (CHEF pattern 1, CHEF pattern 7, CHEF pattern 8, CHEF pattern 17 and CHEF pattern 44) were identified in six countries (Hong Kong, Australia, Singapore, South Africa, Japan and Taiwan). Within these countries several MRSA strains were identified. Although individual strains prominent in some countries, only two strains (CHEFF pattern 7 and 8) were isolated in several Asian countries. CHEF patterns 7 was common in Hong Kong 38 [71%] and Taiwan 7 [13%] but not very common in Singapore 4 [8%], China 3 [6%] and Australia 1 [2%]. The other CHEF pattern 8 was isolated in five countries and was common in Singapore 31 [76%] and less common in Taiwan 5 [12%], Australia 4 [10%] and China 1 [2%]. Plasmid profiles: Some of the isolates (82/309) did not carry any plasmids, however those isolates that carried plasmids varied in sizes with smaller plasmids (2.0-4.0 kb) being disseminated in several countries (Table 3). When transfer of representative plasmids into suitable recipient WBG 1876 by mixed culture transfer and WBG541 and polyethylene glycol respectively, almost all isolates plasmid did not or fail to transfer. Analysis of mecA complex: Majority carried Class A mec complex (Table 4), the strains from South Africa carried Class B mec complex and the variant Class A1 mec complex was identified in one isolate from Hong Kong. The results from the epidemiological methods described demonstrated that the multiresistant MRSA causing bacteraemia, many belonging to bacteriophage Group III, carried small plasmids, have prominent CHEF pattern and carried Class A mec complex appear similar in several hospitals in Southeast Asia and confirm the spread of MRSA causing bacteraemia between Southeast Asia and Australia. While some strains differ considerably in their genomic diversity in the mec complex and are found to be present in hospital such as Japan and South Africa. Three different mec complex classes were found in the randomly selected prominent CHEF patterns isolates (twenty-seven) tested.

DISCUSSION:

MRSA is an important cause of infection in hospitalised patients. It is the third most common pathogen in bacteraemias (43.1%) and the second most common in respiratory tract infections (56.9%) and urinary tract infections (57%) in hospitals [7]. The prevalence of MRSA in blood cultures differs in the countries in this study. Bell et

al., [7] reported that the percentage of MRSA isolates from blood cultures varied from 11.8% in the Philippines, 22.4% in Australia, 26.9% in China, 40.4 in South Africa, 46.7% in Taiwan, 58.2% in Hong Kong, 60.6% in Singapore to 66.8% in Japan. In our present study various methods were used to characterize and type MRSA isolated from bacteraemias in hospitals in these countries. Antibiogram typing of the 309 MRSA isolates from 15 hospitals, revealed that most of the isolates with the exception of isolates from Australia 203 RPH, and three from Hong Kong 204 QMH had multiple-resistant as defined by Pearman et al., [18]. Multiple-resistance is typical of nosocomial MRSA and the resistance pattern in our study is consistent with the findings reported by other researchers [7, 19]. There are some variations in the resistance patterns of the isolates between hospitals and this may reflect the use of antibiotics in these hospitals. Most of the multiple-resistant isolates were resistant to most of the aminoglycosides, macrolides and lincosamide, as well as others such as neomycin, sulphamethoxazole and trimethoprim. Similar patterns of resistance to the aminoglycosides have been reported by others for Australia [20], Hong Kong [6], Japan [19], France [21], Singapore [22] and Kuwait [23]. There were a few notable differences between the isolates from the different countries in the present study. Resistogram typing revealed that most of the isolates were resistant to cadmium acetate. Resistance has been reported as being either chromosomal [24], on ψTn554 inserted in the SCCmec [25] or on a plasmid [13]. Except for the Japanese isolates most of the isolates were sensitive to arsenate. It is interesting that earlier, Classic MRSA were found to be arsenate resistant whereas the later MRSA, EA MRSA, were arsenate sensitive [26]. In most cases the isolates were resistant to both mercury ion (mercuric chloride/HgCl) and organic mercury (phenylmercuric acetate/Pma). Most of the Japanese isolates were sensitive to Pma. The reasons for these differences are not known, but it is unlikely to be due to the use of agents containing these chemicals. Although mercurochromes had been used widely in the past they are rarely used today. There were no clear differences between the isolates from the different countries in their patterns of resistance to ethidium bromide and propamidine isethionate.

Resistance to these two compounds is known as NAB-I [27] or qacA resistance. In a few cases isolates were resistant to one of these but not the other. The reason for this is not known but it has been observed before. Bacteriophage susceptibility demonstrated that the majority of the

isolates were not typable with the IBS phages. This corresponds with the finding of others reported in China [28], Australia [29], Egypt [30], Israel [31] and the United Kingdom [32]. Although common phage lysis patterns were identified in some countries a comparison of the result with the CHEF results shows that phage typing is not as discriminating as the CHEF gel electrophoresis. For example, the 88//M3 phage type is quite common in the Hong Kong isolates and mostly corresponds to CHEF pattern 7 but not all pattern 7 isolates are this phage type and some 88//M3 have quite different CHEF patterns. Also some 88//M3 is found in the Singapore isolates and some of these have CHEF pattern 7. However, some Singaporean CHEF Pattern 7 isolates do not have the 88//M3 phage type. Similarly, some of the Taiwanese isolates have the 88//M3 phage type and are CHEF pattern 7, some CHEF patterns 7s have greatly extended phage lysis patterns and some 88//M3 phage patterns have different CHEF patterns.

Fifty CHEF patterns (Figure 2) were obtained and some were found in more than one country. For example, CHEF pattern 7 was found in five countries and CHEF pattern 8 in four (Table 2). CHEF pattern 1 was found only in the isolates from Australian hospitals and was found in 32 of the 67 (47%) isolates. This pattern corresponds to the US EMRSA 2 that has been found in Sydney and Adelaide hospitals. CHEF patterns 41 and 42 were only found in Singapore isolates and were found in only a few isolates, three and one respectively. Although some isolates from hospitals did not carry any plasmids the majority carried one or more plasmids.

The absence of plasmids in multi-resistant MRSA is uncommon; however, the absence of plasmids has been reported in isolates from Hong Kong [6], China [28] and Portugal [33]. Generally isolates from a hospital contained similar sized plasmids as shown in Table 3, even if their profiles were different, suggesting that plasmids are moving between strains in a hospital. In some cases, as already mention, plasmids of the same size were found in more than one hospital in more than one country.

Small plasmids ranging from 1.7 kb to 3.5 kb were found to be common in isolates from all hospitals. Although isolates may have the same genetic background it has now become apparent that the isolates with the same genetic background can have different SCCmecs (mec regions).

Analysis of the mec complex which consists of an IS431, the mecA gene and its regulatory genes, mecR1 and mecI, have been useful in comparing the relatedness of MRSA [34]. At the time this work was done the mec complexes that had been described in MRSA, were Class A, Class B, Class C

and Class E [35]. Other mec complexes Class A and B are the most common complexes found in MRSA [36]. In this study three different mec complexes (Class A, Class B and Class A1) were detected. The majority of the isolates (23 of 27) had the Class A mec complex. Eleven were CHEF pattern 7, nine CHEF pattern 8 and three CHEF pattern 1. The isolates were from China, Singapore, Taiwan, Hong Kong and Australia. In addition one isolate from Hong Kong had a deletion in the membrane spanning domain of mecR1 and presumably was the same as Class A1.

The widespread occurrence of Class A mec complex in the isolates from these countries is similar to that reported for epidemic strains isolate in England and Australia [38]. Although these results may reflect the overall picture for MRSA in Southeast Asian and Australian hospitals they need to be interpreted with caution because not all isolates were tested; only representatives from the predominant CHEF patterns were tested. However, the results are consistent with other results.

Lim [37] found that many of the Malaysian isolates were the same as the predominant strain in Singapore and that they all had mec Class A with a single base nonsense mutation at nucleotide 202 in mecI. The EA MRSA that was examined had Class A1, that is, it had the same mecI but in addition had a 166 bp deletion in the membrane-spanning domain of mecR1 [37]. Chongtrakool et al. [39] examined isolates from many countries including isolates from Singapore, the Philippines, China and Japan. They found that the majority of the isolates had the Class A mec complex. In fact, with the exception of one isolate from the Philippines, all the isolates from Singapore, the Philippines and China had Class A. The one isolate from the Philippines that was different had the Class B mec and 5.1% of the Japanese isolates were also Class B. Five (3.6%) of the Japanese isolates were found to belonged to an additional mec complex. The three isolates representative of the predominant CHEF pattern 44 in the South African isolates that were isolated had a type B mec complex.

To fully characterize the mec region (SCCmec) it is now necessary to also type the ccr genes and the J (junk) regions, particularly the J1 region, which is now referred to as mec left extremity polymorphism (MLEP) by Chongtrakool et al., [39].

In their recent paper Chongtrakool et al., [39] proposes a new nomenclature for SCCmec typing. It is possible to speculate that in our present study if the isolates were

further typed for the ccr genes and by MLEP typing the isolates may have been found to belong to the additional SCCmec types. Chongtrakool et al., [39] found that most of the Korean and all of the Japanese Class A mec complexes had type two ccr genes whereas the isolates from Singapore, the Philippines and China had type three ccr genes [39]. In addition the isolates could be further divided based on MLEP typing.

Table 1: Common bacteriophage patterns in eight countries

Common bacteriophage patterns										
Countries	No	A	B	C	D	E	F	G	H	I
China	5				1					1
Hong Kong	59			15		2			1	15
Japan	91	8	7		1		5	15	2	47
Philippines	2				1					1
Singapore	43		26	2						6
Taiwan	21		4	5						1
Australia	67	1		1	4	1				5
South Africa	21									3
Total	309	9	37	23	7	3	5	15	3	80

Bacteriophage patterns: A (MR8/M3), C (88/M3), D (56B), E (88), F (MR8/MR12) and I (non-typable)

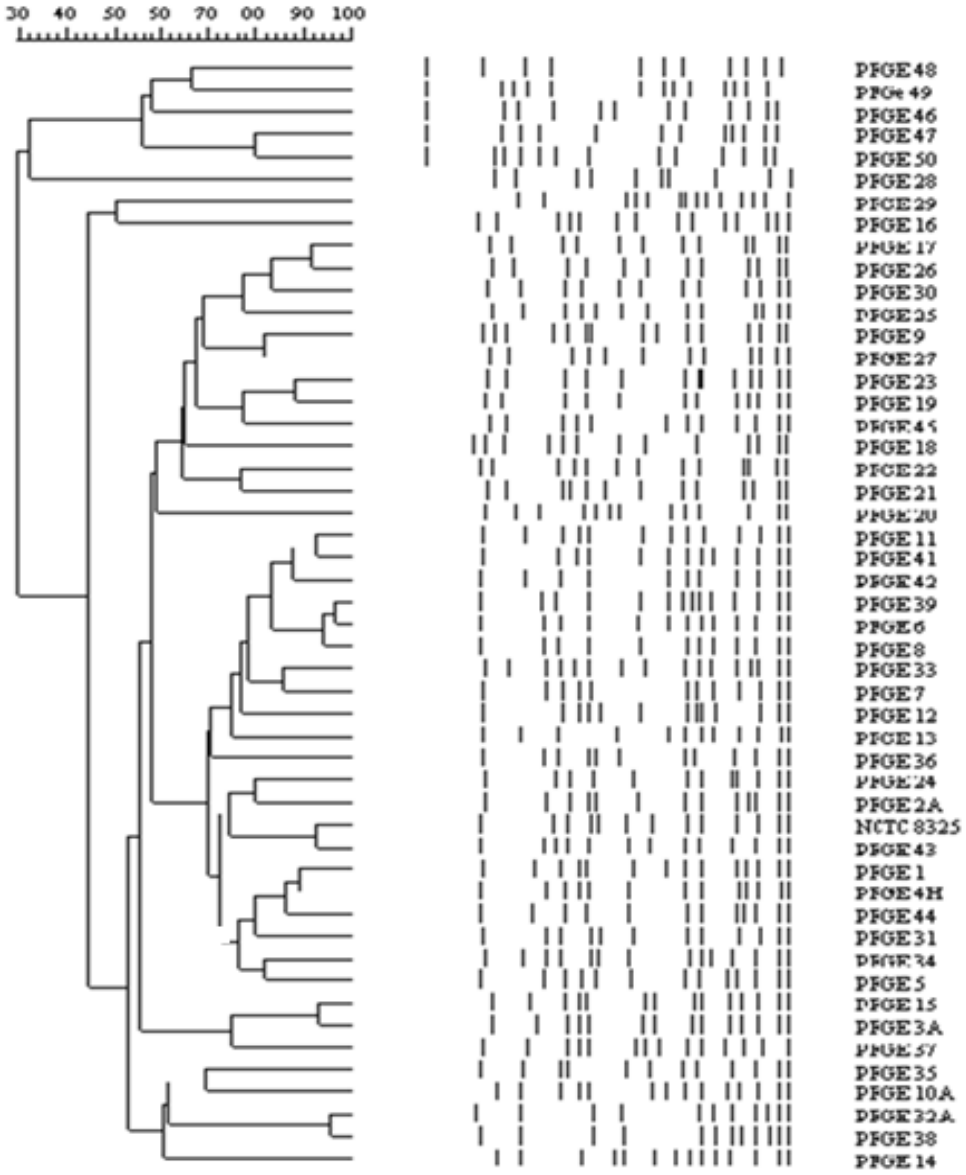

Figure 1: Dendrogram of 50 pulsotypes CHEF patterns for the MRSA

Table 2: Predominant CHEFF pattern

Predominant CHEFF Pattern		1	7	8	17	44
Country	MRSA 309	(32)	(53)	(40)	(15)	(18)
Isolates						
China	5	--	3 [6%]	1 [2%]	--	--
Hong Kong	59	--	38 [71%]	--	--	--
Japan	91	--	--	--	15 [100%]	--
Philippines	2	--	--	--	--	--
Singapore	43	--	4 [8%]	30 [76%]	--	--
Taiwan	21	--	7 [13%]	5 [12%]	--	--
Australia	67	32 [100%]	1 [2%]	4 [10%]	--	--
South Africa	21	--	--	--	--	18 [100%]

Table 3: Predominant plasmid size patterns (kb)

Country	Size (kb)	Predominant plasmid size patterns (kb)
China	18.5 and 3.3	3.2
Hong Kong	32.0 and 2.6	3.2
Japan	40.3 and 1.6 35, 23	2.6
Philippines	Did not carry any plasmid	
Singapore	31.6 and 2.0	3.0
Taiwan	25.5 and 1.7	2.2, 1.9, 1.7
Australia	39.5 and 2.0	3.2, 2.7, 2.4
South Africa	39.1 and 3.0	35.1

Table 4: Results for the amplification of the *mec* complex

Isolate		CHEF pattern	*mecA*	*mecR1* MS	*mecR1* PB	*mecI*	IS*1272*
Class A *mec* complex							
AR19	Australia	8	+	+	+	+	-
S1	Singapore	8	+	+	+	-	
S2	Singapore	8	+	+	+	-	
S28	Singapore	8	+	+	+	-	
S17	Singapore	8	+	+	+	-	
S5	Singapore	8	+	+	+	-	
S21	Singapore	8	+	+	+	-	
CG1	China	8	+	+	+	+	-
TV1	Taiwan	8	+	+	+	+	-
AR1	Australia	1	+	+	+	+	-
AA1	Australia	1	+	+	+	+	-
AP2	Australia	1	+	+	+	+	-
AA6	Australia	7	+	+	+	+	-
S7	Singapore	7	+	+	+	-	
S22	Singapore	7	+	+	+	-	
HK16	Hong Kong	7	+	+	+	+	-
HK3	Hong Kong	7	+	+	+	+	-
HK19	Hong Kong	7	+	+	+	+	-
HK11	Hong Kong	7	+	+	+	+	-
HK47	Hong Kong	7	+	+	+	+	-
HK57	Hong Kong	7	+	+	+	+	-
TN3	Taiwan	7	+	+	+	+	-
TC3	Taiwan	7	+	+	+	+	-
Variant Class A *mec* complex							
HK7	Hong Kong	7	+	-	+	+	-
Class B *mec* complex							
SA1	South Africa	44	+	+	-		+
SA4	South Africa	44	+	+	-		+
SA18	South Africa	44	+	+	-		+

Abbreviation: MS, membrane spanning domain; PB, penicillin binding domain;
-, not amplified; +,amplified

CONCLUSION:

Most of the MRSA were resistant to multiple antimicrobials which is a problem for treatment because only vancomycin is use to reliably treats staphylococcal infection without a laboratory report where MRSA is endemic. Few Hong Kong isolates were non-multi-resistant and were suggestive of community MRSA. Most of the isolates were not typable with IBS phages in contrast to IMS phages. Although some Hong Kong and few Taiwan as well as Singapore isolates have IMS phage pattern 88/M3 that have CHEF pattern 7, there was generally little correlation with phage typing. PFGE is not suitable for comparing isolates between different hospitals and countries and over long time periods but the value of this test is suitable for studying outbreaks in hospitals. It was not possible to characterise many of the plasmids isolated and generally the plasmid profiles of the isolates were characteristic of a particular country. Except for the South African isolates, which had type B *mec* complex, all isolates tested had

type A *mec* complex while the Hong Kong isolate had type A1 *mec* complex.

Our results indicate that there is no particular strain that was responsible for bacteraemias in the countries studied. Each country appears to have its own predominant strain or strains.

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