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Characterization of *Staphylococcus aureus* Isolated from the Skin Surface of Athletes and Training Environment by Random Amplified Polymorphic DNA and Antibiotic Resistance Profiling

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Abstract: *Staphylococcus aureus* isolated from the skin surface of the athletes and their training environment in Malaysia were characterized for their antimicrobial susceptibility and Random Amplified Polymorphic DNA (RAPD). A total of 19 types of antibiotics used in this study and all the isolates were resistant to nalidixic acid and trimethoprim and showed Multiple Antibiotic Resistant (MAR) indexes, ranging from 0.11 to 0.68. No methicillin-resistant *Staphylococcus aureus* (MRSA) was found among the isolates tested in this study. The dendrogram obtained from the results of the RAPD-PCR discriminated the isolates into 4 clusters and 30 single isolates at the level of 100% similarity. Isolates from different individual and the environment were clustered together and colonized by similar strains.

Key words: RAPD-PCR, *Staphylococcus aureus*, athletes, antibiotic resistance

INTRODUCTION

S. aureus is the causative agent of many opportunistic infections in human and animals (Kools and Bannerman, 1995). As a human pathogen, *S. aureus* causes superficial, deep-skin and soft tissue infections, endocarditis and bacteremia, as well as a variety of toxin-mediated diseases including gastroenteritis, staphylococcal scalded-skin syndrome and toxic shock syndrome (Fidalgo *et al.*, 1990; Roberts *et al.*, 1991). In Malaysia, outbreaks of *S. aureus* infection among athletes have rarely been reported. According to Stacey *et al.* (1998), outbreaks of cutaneous infection among participants of physical contact sports such as rugby football are well recognized and documented in the medical literature.

There are two basic mechanisms responsible for the resistance of *S. aureus* to the beta-lactam antimicrobial agents. The first is the production of beta-lactamases and the mechanism is the presence of low affinity penicillin-binding proteins that made them resistant to the antistaphylococcal penicillins including methicillin. *S. aureus* can develop resistance to antibiotics with amazing efficiency (Mulligan *et al.*, 1993).

An increasing number of reports have indicated that application of the Polymerase Chain Reaction (PCR) to reliably and quickly detect pathogens (Benito *et al.*, 2000). Arbitrarily primed-PCR (AP-PCR) allows larger proportions of the genomes studied in generation of the banding patterns, allowing finer discrimination among strains (Welsh and McClelland, 1990; Williams *et al.*, 1990).

The aim of this study was to determine the relatedness of *S. aureus* strains isolated from the skin surface of the healthy athletes with the training environment and the antimicrobial susceptibility profile. Typing of *S. aureus* strains by phenotypic markers is difficult because of the variation in results (Tenover *et al.*, 1994). Therefore we have chosen to study the DNA patterns (genotype) generated by a PCR using primers based on repetitive sequences in the DNA (van Belkum *et al.*, 1993).

MATERIALS AND METHODS

Bacterial strains: This study was carried out on 44 isolates of *S. aureus* of which 16 isolates were isolated from the skin surface of outdoor athletes, 18 from the skin

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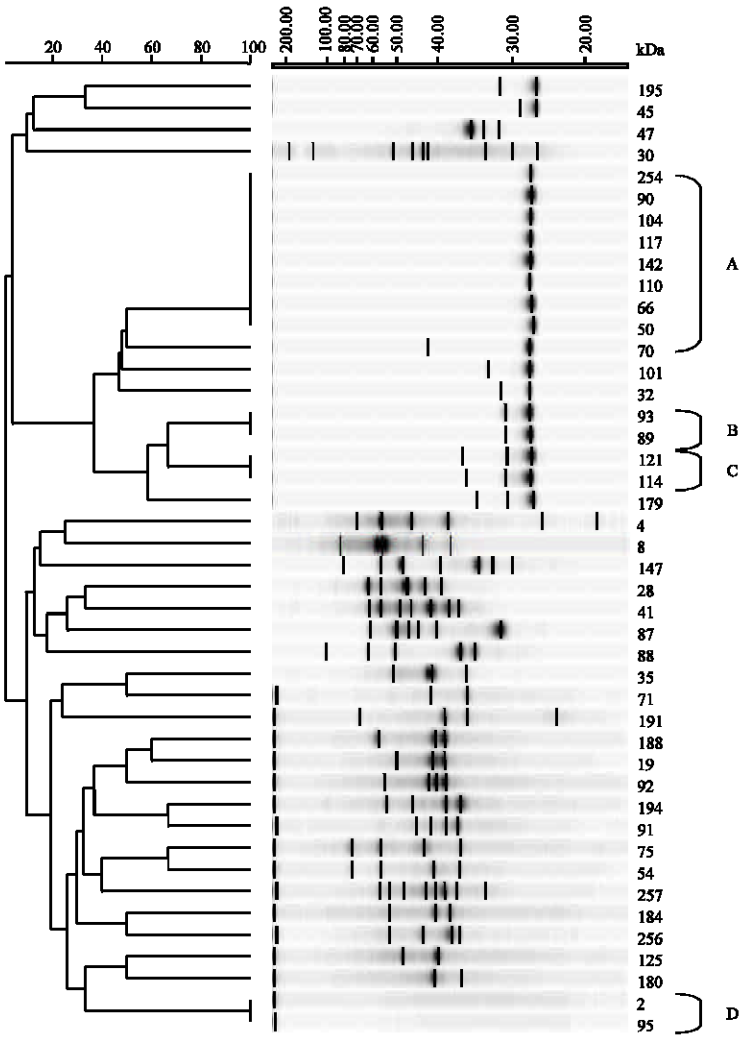

Fig. 1: Dendrogram generated from the RAPD-PCR analysis using primer GEN1-50-01 (5'GTGCAATGAG-3') of the *Staphylococcus aureus* strains. A-D: clusters generated at 100% similarity.

surface of indoor athletes and 10 from the surrounding training environment of school field, Petaling Jaya, Malaysia. They were 4 isolates from the outdoor and 6 isolates were from the indoor in the training environment of school field, Petaling Jaya, Malaysia.

Antibiotic susceptibility testing: Disk diffusion tests were performed with antibiotic containing disks obtained from BBL (Becton Dickinson, USA) Microbiology System, Cockeysville, MD, by the method recommended by the National Committee for Clinical Laboratory Standards (NCCLS). The antimicrobial agents tested included Norfloxacin (10 μg), Doxycycline (30 μg), Nitrofurantoin (300 μg), Tetracycline (30 μg), Nalidixic Acid (30 μg), Rifampicin (30 μg), Neomycin (10 μg), Imipenem (10 μg), Streptomycin (10 μg), Chloramphenicol (30 μg), Trimethoprim (25 μg), Ciprofloxacin (5 μg), Methicillin (1 μg), Gentamicin (10 μg), Ampicillin (10 μg), Cephalothin (30 μg), Vancomycin (30 μg), Clindamycin (2 μg) and Oxacillin (1 μg). Bacteria were suspended in saline to the same visually as the Mc Farland 0.5 turbidity standard and streaked on Mueller Hinton agar. Plates were incubated for 24 h at 37°C. Characterization of strains as sensitive, intermediate or resistant was based on the size of zones of inhibition surrounding the discs. The Multiple Antibiotic Resistance (MAR) index was calculated according to Krumperman (1983) using the following formula: MAR index = x/y, where x is the number of resistance determinants and y is the number of antibiotics tested.

DNA Preparation and RAPD analysis: Prior to amplification by PCR method, chromosomal DNA of *S. aureus* isolates were extracted by the mini-preparation

method by Williams *et al.* (1990). Ten randomly designed 10-mer primers with 50% G+C content (Genosys Biotechnologies Inc, USA), designated as GEN1-50-01 to GEN1-50-10 were screened and only one primer, the GEN1-50-01 (5'GTGCAATGAG-3') was selected for the RAPD analysis as they provide reproducible and discriminatory banding patterns. PCR reactions for the RAPD assays were performed in 25 μL volumes containing 20 ng of genomic DNA, 2.5 μL of 10x PCR buffer, 0.5 μL of 10 mM dNTPs, 1.5 μL of 25 mM $MgCl_2$, 1 unit of *Taq* polymerase (Promega Co, USA) and 5 pmol of primer. The Amplifications were carried out in a thermal cycler (Perkin Elmer 2400). The cycling parameters were 4 min at 94°C for pre-denaturation, 45 cycles each of 1 min at 94°C for denaturation, 1 min at 36°C for annealing, 2 min at 72°C for extension and a final extension at 72°C for 8 min. The PCR amplification products were visualized by running 15 μL of the amplification products on 1.2% agarose gel, which was stained with ethidium bromide (0.5 μg mL^{-1}) and photographed under UV transilluminator. The RAPD assays mentioned above were repeated three times to determine the reproducible of the banding patterns generated.

RAPD fingerprint pattern analysis: The banding profiles generated by RAPD-PCR were analyzed with GelCompar software (Version 4.1, Applied Maths, Kortrijk, Belgium). The arrangement of the RAPD-PCR profiles into the dendrogram was accomplished by the unweighted pair group method with arithmetic averages (UPGMA) using Jaccard co-efficient.

RESULTS

Antibiotic resistance tests showed that *S. aureus* isolates were resistance to most of the antibiotic tested (Table 1). The multiple antibiotics resistant (MAR) index ranged from 0.11 to 0.68 as shown in Table 1. All the *S. aureus* isolates showed 100% resistance to nalidixic acid and trimethoprim. This was followed by resistance to streptomycin (68.2%), clindamycin (61.4%), gentamicin (52.3%), neomycin (50%), ampicillin (50%), tetracycline (43.2%), chloramphenicol (36.4%), ciprofloxacin (31.2%), doxycycline (13.6%), vancomycin (13.6%) and nitrofurantoin (6.8%). Among all the antibiotics tested, norfloxacin, cephalothin, imipenem, rifampicin, methicillin and oxacillin gave total inhibition of all the isolates tested.

The profiles obtained from the RAPD contained 1 to 9 bands within the molecular size range of 0.3 to 5.0 kbp. Using the Gel Compar Ver 4.1 image analysis software, the forty-four isolates tested were separated into 4 major clusters (A-D) and 30 single isolates at 100% similarity level (Fig. 1) by Jaccard coefficient of comparison.

DISCUSSION

Knowledge of the normal microbial population of the skin surface has been established as an important issue in the pathophysiology of skin disease in human beings and animals (Lilenbaum *et al.*, 1998). It is believed that the normal microbial population inhibits the proliferation of pathogenic organism by depriving them of nutrients, as well as the presence of bacteriocins, antagonism, occupation and adhesions and other competition systems. It is usually harmless, but after predisposing factors such as trauma or concurrent infection, some of these organisms may become potential pathogens, multiplying and causing bacterial dermatitis.

In mammals, staphylococci are found primarily on the skin and possibly, on the anterior nare (Kloos, 1990). In addition, *S. aureus* is the most important staphylococcal species in man (Lilenbaum *et al.*, 1998). On the other hand, there is no study being conducted so far to monitor the relationship between distribution of *S. aureus* on the skin of a healthy athlete with the training environment and the performances.

The development of antimicrobial resistance nearly always has followed the therapeutic use of antimicrobial agents. Since antibiotic use became widespread 50 years ago, bacteria have steadily and routinely developed resistance. Therefore, monitoring of normal microbial towards antibiotic resistance serve an important issue in relation of emergence pathogens.

Testing for oxacillin resistance in staphylococci has been a challenge for clinical laboratories for more than 15 years (McDougal and Thornsberry, 1984). Oxacillin is more resistant to degradation in storage and is widely used for the treatment of *S. aureus* infection. In this study, all the isolates tested against oxacillin shows sensitivity. Thus, we suggest that all the isolated *S. aureus* does not carries an altered penicillin-binding protein, PBP2a, which is encoded by the *mec*A gene. The alteration of the penicillin-binding protein does not allow the drug to bind well to the bacterial cell, causing resistance to β-lactam antimicrobial agents.

Worldwide, many strains of *S. aureus* are already resistance to all antibiotics except vancomycin (Mansouri and Khaleghi, 1997) and thus the organism has progressed one step closer to becoming an unstoppable killer. In our study, only *S. aureus* strains isolated from the healthy skin of outdoor athlete indicate total sensitivity against vancomycin. A total of 5 isolates obtained from indoor athletes demonstrated sensitivity towards vancomycin tested. Only one single isolate showed sensitivity against vancomycin was detected

Table 1: Antibiogram, plasmid size and MAR index of *Staphylococcus aureus* strains isolated from various sources

	Strain No.	Antibiogram	Plasmid size (megaDalton)	MAR index
Skin surface of the athletes during outdoor training.				
	101	TE30,NA30,N10,RL25,GM10,AM10,C30,CC2	ND	0.42
	104	DO30,TE30,NA30,N10,S10,RL25,CIP5,GM10,AM10,C30,CC2	1.8	0.58
	110	NA30,N10,S10,RL25,GM10,C30,CC2	2.6, 1.8	0.37
	114	TE30,NA30,N10,S10,RL25,GM10,AM10,C30,CC2	ND	0.47
	117	NA30,RL25,AM10	ND	0.16
	121	NA30,S10,RL25,CIP5,GM10,AM10	ND	0.32
	125	TE30,NA30,S10,RL25,GM10,AM10,CC2	ND	0.37
	142	NA30,RL25,GM10	ND	0.16
	147	NA30,RL25,	ND	0.11
	179	NA30,RL25,AM10	ND	0.16
	180	DO30,NA30,S10,RL25,AM10	2.6	0.26
	184	NA30,RL25,AM10	ND	0.16
	188	TE30,NA30,S10,RL25,GM10,AM10,C30,CC2	ND	0.42
	191	NA30,RL25,AM10	ND	0.32
	194	NA30,RL25	ND	0.11
	195	NA30,RL25,AM10	ND	0.16
Skin surface of the athletes during indoor training.				
	2	DO30,F300,TE30,NA30,N10,S10,RL25,CIP5,GM10,AM10,C30,VA30,CC2	ND	0.68
	4	F300,TE30,NA30,N10,S10,RL25,CIP5,GM10,AM10,C30, CC2	ND	0.58
	8	F300,TE30,NA30,N10,S10,RL25,CIP5,GM10, AM10,C30,VA30,CC2	ND	0.63
	19	DO30,TE30,NA30,N10,S10,RL25,CIP5,GM10, AM10,C30,CC2	ND	0.58
	28	DO30,TE30,NA30,N10,S10,RL25,CIP5,GM10, AM10,C30,CC2	ND	0.58
	30	NA30,N10,S10,RL25,CIP5,GM10,C30,CC2	ND	0.42
	32	DO30,TE30,NA30,N10,S10,RL25,AM10	2.6	0.37
	35	TE30,NA30,RL25	ND	0.16
	41	TE30,NA30,N10,S10,RL25,CC2	ND	0.32
	45	TE30,NA30,N10,S10,RL25,CIP5,GM10,C30,VA30,CC2	2.0	0.53
	47	NA30,S10,RL25	ND	0.16
	50	NA30,N10,S10,RL25,CIP5,GM10,C30,VA30,CC2	ND	0.47
	54	TE30,NA30,N10,S10,RL25,CIP5,GM10,C30,CC2	3.4, 2.6	0.47
	66	NA30,N10,S10,RL25,CIP5,VA30,CC2	ND	0.37
	70	NA30,S10,RL25,CIP5,CC2	3.4, 2.6	0.26
	71	TE30,NA30,N10,S10,RL25,GM10,CC2	3.4, 2.6	0.37
	75	NA30,N10,S10,RL25,GM10,CC2	2.6	0.32
	87	NA30,N10,S10,RL25,GM10,CC2	ND	0.32
Environment				
Indoor	88	NA30,RL25	ND	0.11
Indoor	89	NA30,S10,RL25,GM10,CC2	ND	0.26
Outdoor	90	TE30,NA30,N10,S10,RL25,GM10,AM10,CC2	ND	0.42
Indoor	91	TE30,NA30,S10,RL25,VA30,CC2	2.6	0.32
Indoor	92	NA30,N10,S10,RL25,CIP5,C30,CC2	ND	0.37
Indoor	93	TE30,NA30,N10,S10,RL25,GM10,CC2	2.6, 2.0	0.37
Indoor	95	NA30,S10,RL25,AM10,C30,CC2,	ND	0.32
Outdoor	257	NA30,RL25	2.0, 1.8	0.11
Outdoor	254	NA30,RL25,AM10	2.0, 1.8	0.16
Outdoor	256	NA30,RL25,AM10	ND	0.16

Symbols for antimicrobial resistance: NOR, Norfloxacin; DO, Doxycycline; F, Nitrofurantoin; TE, Tetracycline; NA, Nalidixic Acid; RD, Rifampicin; N, Neomycin; IPM, Imipenem; S, Streptomycin; C, Chloramphenicol; RL, Trimethoprim, CIP, Ciprofloxacin; MET, Methicillin; GM, Gentamicin; AM, Ampicillin; KF, Cephalothin; VA, Vancomycin; CC, Clindamycin; OX, Oxacillin; ND = none detected

Table 2: Common resistant and susceptible profile based on the clusters generated from dendrogram generated

Cluster	Common resistant profile	Common susceptible profile
A	-	F300
B	NA30,S10,RL25,GM10,CC2	NOR10,DO30,F300,IPM10,CIP5,MET1,AM10,KF30,VA30,OX1
C	NA30,S10,RL25,GM10,AM10	NOR10,DO30,F300,RD30,IPM10,MET1,KF30,VA30,OX1
D	S10, AM10, C30, CC2	-

from the indoor environment. Thus, it suggests there is a relationship between the environment and the skin of the indoor athletes.

The major differences in the antibiotic resistance profile obtained between three major categories studied were sensitivity of vancomycin among all *S. aureus* isolated from outdoor athletes and doxycycline among all environmental isolates. The number of the isolates obtained from indoor athletes resistant against vancomycin and doxycline were 5 and 4, respectively. Both environmental isolates and outdoor athletes' isolates show total sensitivity against nitrofurantoin

where as only 3 indoor athletes isolates demonstrated resistance against nitrofurantoin. Since vancomycin inhibits bacterial cell wall synthesis and doxycycline inhibit bacterial
protein synthesis, both antimicrobial-tested enable us for further research on both category isolates mentioned.

The low Multiple Antibiotic Resistance (MAR) index value (0.11-0.68) suggested that isolated *S. aureus* were unlikely to have a predisposition to develop resistance under conditions of antibiotic selective pressure. Multiple antibiotic resistance can occur even in the absence of plasmid or transposon. Brown *et al.* (1991) suggested that under laboratory conditions, the absence of antibiotics in the culture media probably enhance plasmid instability.

Generally, antibiotics such as norfloxacin, cephalothin, imipenem, rifampicin, methicillin, oxacillin, nalidixic acid and trimethoprim that were used in this study were not included in the cluster analysis since all the isolated demonstrated the same resistance profile. According to the dendrogram obtained, all the isolates were clustered into 2 main cluster at 0% similarity with one cluster indicate total sensitivity towards nitrofurantoin (Cluster A, Table 2). Each cluster generated showed a unique resistant and susceptible profile (Table 2). Besides that, Cluster A (Fig. 1) generated consist of isolates from different sources such as athletes and the environments that indicate the colonization profiles of the *S. aureus*.

The uniqueness profile of each cluster indicates the discriminatory power of RAPD and the characterization ability. Thus, the use of nucleic acid amplification by PCR has applications in many fields, especially characterization of bacteria. Additionally, RAPD-typing may provide a more rapid and reliable method to distinguish between high and low virulence *S. aureus* strains (Hermans *et al.,* 2000). Moreover, RAPD-typing is simpler to execute and interpret compared to other genotyping methods, such as genomic restriction endonuclease Fingerprinting and DNA hybridization (Matthews *et al.,* 1994). Besides that, the rapidity of the method and its cost-effectiveness (van Belkum *et al.,* 1995) make it suitable for use as a preventive diagnostic test

The results of this study indicate that the *S. aureus* strains are highly diverse, despite the fact that many of the isolates share the same antibiotic resistance profile. Indirectly, this may suggest that the resistance patterns are not associated with major genetic alterations and that the diversity observed may attribute by other factors. Therefore, RAPD fingerprinting technique has great potential for the characterization of environmental isolates that may experience genomic changes before or after the resistance has developed.

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