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Phylogenetic Diversity of *Staphylococcus aureus* by Random Amplification of Polymorphic DNA

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Abstract: The polymerase chain reaction was used to obtain randomly amplified polymorphic DNA profiles for genetic fingerprinting of twelve different isolates of *Staphylococcus aureus* from Riyadh region, KSA using RAPD-PCR technique eleven out of forty operon primers (10-mer primers) showed polymorphism among the isolates tested generating over 65 bands, approximately 42 of which were polymorphic with sizes ranging between 300 and 3,000 bp. A numerical analysis of the genomic profiles obtained demonstrated that it was possible to differentiate the *S. aureus* strains. Using this technique. All the isolates were classified completely into three major groups (Sc.A, Sc.B, Sc.C) with five different subgroups. Sc. A group originated from animal host while isolates from plant and animal origins formed the Sc.B and Sc.C group. The five different subgroups of Sc. A suggested adaptation of *S. aureus* in different host cells. This indicates possible relationships between host origin and genetic variation among *S. aureus* isolates. The DNA fingerprint defined for each race of *S. aureus* could be useful in epidermiological studies, medical diagnosis and the identification of new strains and their origins.

Key words: *Staphylococcus aureus*, genetic fingerprinting; phylogenetic diversity, RAPD, polymorphism; DNA amplification; DNA ladder

INTRODUCTION

Staphylococcus aureus is one of the most common caused of food borne-acquired infections, causing a wide variety of infections, from simple absecessed to fatal sephsis, as well as endocarditis, meningitis and toxinoses including food poisoning and toxic shock syndrome. Staphylococcus pathogenic versatility is compounded by its ability to develop resistance to new antibiotics almost as fast as they are introduced. However, nosocomial infections caused by *S. aureus* are clinically serious and control of such infection requires strain typing to identify degree of virulence, the source of contamination, and resistance to commonly used antibiotics.

It is important in epidemiology and ecology to be able to identify bacterial species and strains accurately. Rapid identification and classification of bacteria is normally carried out by morphology, nutritional requirements, antibiotic resistance, isoenzyme comparisons, phage sensitivity (Eisenstein, 1990; Selenader *et al.,* 1987; Aber and Mackel, 1981; Milkman, 1973) and more recently by DNA based methods, particularly rRNA sequences (Woese, 1986), strain-specific fluorescent oligonucleotides (Delong *et al.,* 1989; Amann *et al.,* 1990) and the polymerase chain reaction (PCR) (Mullis and Faloona, 1987; Smith and Selander, 1990; McCabe, 1990). PCR-based methods have an advantage over some other molecular techniques as they can produce rapid and reliable results.

Detection and identification methods using the PCR to amplify DNA have been used for other organisms (Hartskeeri *et al.,* 1989), but these require sequence information for specific primers. However, PCR using arbitrary primer (AP-PCR) requiring no prior sequence information has revealed DNA polymorphisms that may be used for fingerprinting (Welsh and McClelland, 1990; Williams *et al.,* 1990). The randomly amplified polymorphic DNA (RAPD)-PCR technique is a modification of the polymerase chain reaction which can be used to produce genome fingerprints of the strains under examination. Random amplified polymorphic amplification of discrete DNA fragments in the genome by the use of oligonucleotide primers with random

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sequences, have been largely used to identify physiological races of fungi (Guthrie *et al.,* 1992). With this technique a DNA fingerprint may define individual in a very fast and reliable way. RAPD-PCR method, when compared with biochemical methods is cheap, simple, more sensitive and faster. Apart from the study of antibiotic resistance (Ikeh, 2003), little is known concerning the genetic diversity that exists in populations of *S. aureus* isolated from human and food origins in Saudi Arabia.

In this study genetic fingerprinting and phylogenetic diversity of isolates of *S. aureus* from different sources in Riyadh region, Kingdom of Saudi Arabia was evaluated using RAPD markers. Such information will be useful in its classification,epidemiological survey,ecology and diagnosis.

MATERIALS AND METHODS

Bacterial Isolation:

A total of 60 strains were isolated from human to food origin in Riyadh region, KSA. Twelve strains (Table 1) out of the 60 strains were their identity had been confirmed by susceptibility to antibiotics and coagulase biochemical test isolates preservation and storage were in accordance with Gore and Walsh (1964). Staphylococcus isolates were first propagated using a modified procedure developed by Kado and Keskett (1970). About 250 ml *S. aureus* isolate was transferred into 100 ml of nutrient broth (pH 7.5) and kept under constant shaking at 37°C for 24 h. The bacterial cell was removed by centrifugation, washed with 0.1mM Tris-EDTA and kept at -20°C for DNA extraction.

Genomic DNA Extraction:

DNA extraction was according to Roeder and Broda (1987) and Thottappilly *et al.* (1999) with some modification 0.3 g of washed bacterial cell were suspended in 200 ml of 2xCTAB buffer (50 mM Tris, pH 8.0; 0.7 mM NaCl; to mM EDTA; 2% hexadecyltrimethylammonium bromide; 0.1% 2-mercaptoethanol), followed by the addition of 100 ml of 20 % sodium dodecyl sulfate and incubated at 65°C for 10 min. DNA was purified by two extraction with phenol:Chloroform:isoamyl alcolhol (24:25:1) and precipitated with -20$_o$C absolute ethanol. After washing with 70% ethanol, the DNA was dried and resuspended in 20 ml of sterile distilled water. DNA concentration was measured using DU-65UV spectrophotometer (Beckman Instruments Inc., Fullerto CA, USA) at 260 nm. DNA degradation was checked by electrophoresis on a 1% agarose gel 1xTAE (45 mM Tris-acetate, 1 mM EDTA, pH 8.0).

Random Amplified Polymorphic DNA Fingerprinting:
(RAPD-PCR analysis):

RAPD-PCR analysis was according to Guthrie *et al.* (1992). DNA primers tested were purchased from Operon Technologies (Alameda, California, USA) and each is 10 nucleotides long. Two concentrations of each DNA (24 ng and 96 ng per reaction) were used to test reproducibility and eliminate sporadic amplification products from the analysis. Forty primers (opx and opy) wee screened with isolates for their ability to amplify the *S. aureus* DNA eleven of these primers (Table 2) were found useful since they gave polymorphism. These were used in amplifying the DNA from all *S. aureus* isolates. Amplifications were performed in 25 ml reaction mixture consisting of genomic DNA, 1X reaction buffer (Promega), 100 mM each of dATP, dCTP, dGTP, and dTTP, 0.2 mM Operon random primer, 2.5 mM MgCl2 and 1U of Taq polymerase (Boehringer, Germany). A single primer was used in each reaction. The reaction mixture was overlaid with 50 ml of mineral oil to prevent evaporation. Amplification was performed in a thermowell microtiter plate (Costa Corporation) using a Perkin Elmer programmable Thermal controller mode 9600. The cycling program was (i) 1 cycle of 94°C for 3 min; (ii) 45 cycles of 94°C for 1 min for denaturation, 40oC for 1 min for annealing of primer and 72°C for 2 min for extension; and (iii) a final extension at 72°C for 7 min. The amplification products were resolved by electrophoresis in a 1.4% agarose gel using TAE buffer (45 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 100 V for 2 h. A 1 kb ladder (Life Technolgies, Gaithersburg, MD, USA) was included as molecular size marker. Gels were visualized by staining with ethidium bromide solution (0.5 mg/ml) and banding patterns were photographed over UV light using a red filter.

Phylogenetic Analysis:

Positions of unequivocally scorable RAPD bands were transformed into a binary characters matrix ("1" for the presence and "0" for the absence of a band at a particular position). Pairwise distance matices were complied by the NTSYS-pc 2.0 software (Rohlf, 1993) using the Jaccard coeffieicnt of similarity (Jaccard, 1908). Phylogenetic tree was created by the unweighted pair-group method arithmetic (UPGMA) average cluster analysis (Sneath and Sokal, 1973; Swofford and Olsen, 1990).

RESULTS AND DISCUSSION

Results:

The genomic fingerprints derived from the RAPD analysis of the strains studied are shown in Fig. 1, 2. Eleven primers showed polymorphism among individuals isolates out of 40 primers tested. The amplification reactions with the 11 primers generated up to 105 bands, 50 of them being polymorphic (Table 2) with sizes ranging between 200 and up to 2500 base pairs (Fig 1 and Fig 2). Up to 9 bands were observed in each

Table 1: Isolates of *Staphylococcus aureus* in this study

Strain Number	Isolate Code	Host	Source	Locality of the source
1	Sc1	Cow	Cooked Meat	Farm1
2	Sc2	Cow	Cooked Meat	Farm2
3	Sc3	Cow	Cow Milk	Farm1
4	Sc4	Commericial	Milk	Riyadh
5	Sc5	Commericial	Milk	Riyadh
6	Sc6	Commericial	Milk	Riyadh
7	Sc7	Soybean	Soyamilk	Riyadh
8	Sc8	Soybean	Soyamilk	Riyadh
9	Sc9	Soybean	Soyamilk	Riyadh
10	Sc10	Soybean	Soyamilk	Riyadh
11	Sc11	Human	Urine	Riyadh
12	Sc12	Human	Urine	Riyadh

Table 2: Oligonucleotide primers that showed genetic discrimination among the S. aureus isolates using RAPD-PCR analysis.

Operon code	Nucleotides sequence 5' to 3'	No. of fragments amplified	No. of polymorphic brands
OPX-04	CCGCTACCGA	12	6
OPX-12	TCGCCAGCCA	14	9
OPX-15	GAGGCCAGGA	15	9
OPX-17	GACACGGACC	17	8
OPX-20	CCCAGCTAGA	7	5
OPX-01	GGTGGCATCT	8	3
OPX-07	CTGGACGTCA	5	3
OPX-09	GTGACCGAGT	7	5
OPX-10	TCGCATCCCT	6	2
OPX-11	CTGATGCGTG	6	3
OPX-13	CACAGCGACA	8	6
	Total	105	59

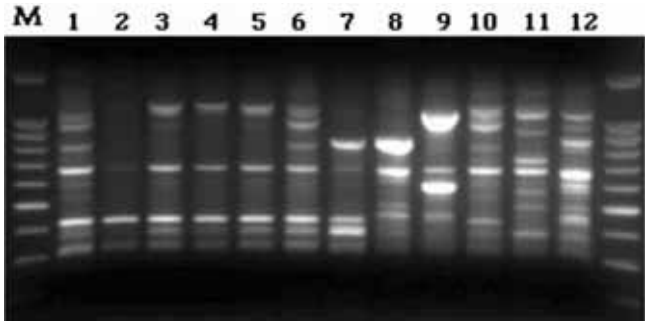

Fig. 1: DNA fingerprinting patterns of 12 *S. aureus* isolated using opx - 15 RAPD primer M = 100bp DNA.

fingerprint and they are within the 25oo bp and 200 bp size range. In all cases, the RAPD banding patterns were reproducible in duplicated experiments strongly amplified DNA segments were consistently observed although there was evidence of minor variations in the band intensity. There are number of bands were common to all twelve strains. However, strain differences that are evident by visual examination of the RAPD patterns were clarified by computerized cluster analysis of the data. The numerical analysis of the patterns revealed three multimembered clusters with similarity levels at or above 80% (Fig. 1). Using 50 RAPD markers to construct phylogenetic relationship among twelve *S. aureus* isolates led to classification into three major cluster groups (A, B and C) defined at 70% similarity level were also recognized. Cluster group A contained strain Sc1, 3, 4, 5 12; cluster group B contain Sc 10, 11, 12, 7 and 8, while cluster group C contain Sc 8, 1, 12, 7, 3, 5, 2 and 4. There similarity of 100% level were recognized in similarity coefficient Fig. 3.

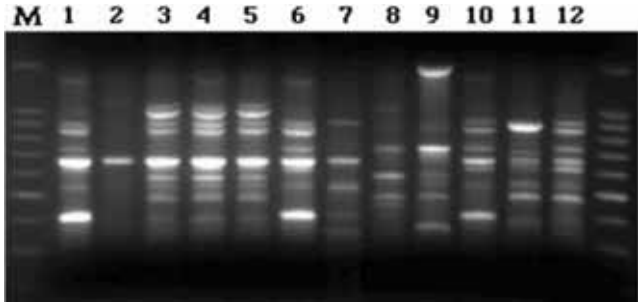

Fig. 2: DNA fingerprinting patterns of 12 *S. aureus* isolated using opx - 17 RAPD primer M = 100bp DNA.

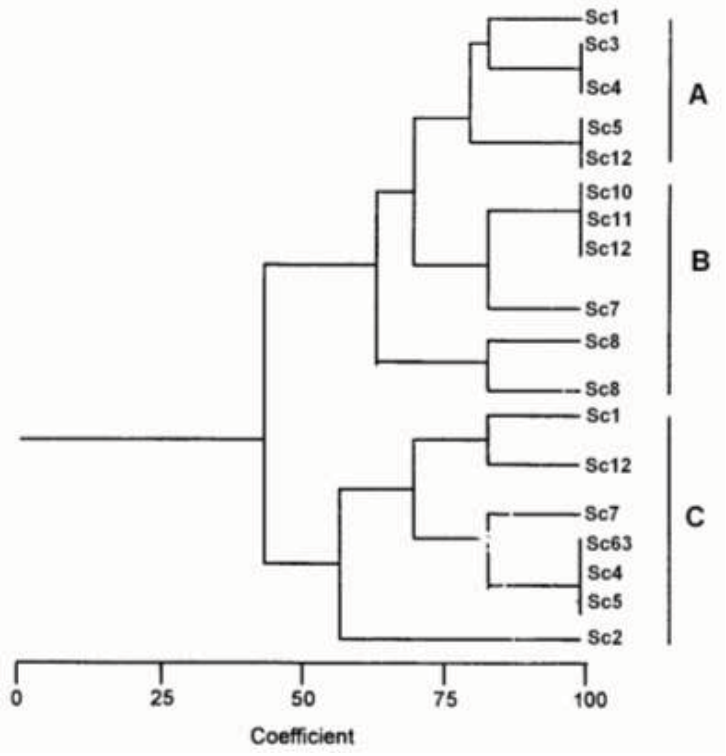

Fig. 3: Dendogram analysis showing phylogenetic diversity of 12 *Staphylococcus* isolates identified by 69 RAPD markers

Discussion:

In recent years the RAPD-PCR technique has been reported as being particularly suitable for typing both within and between bacterial species (Olubukola O. Babalola, 2003).

Genetic fingerprinting and phylogenetic diversity between different *S. aureus* isolates were determined by converting RAPD data into a Jaccard similarity matrix and analyzed by UPGMA to produce a phylogenetic tree. The DNA band pattern obtained is similar to a bar code, allowing the identification of each individual. For instance, isolate Sc 7 presents unique band when its DNA amplified with most of the primers tested (Fig. 1). These bands could be used to characterize and identify it. All the isolates were classified completely into three major groups ScA,ScB,ScC. ScC group comprised of isolates originated from human while isolates from plant and animal origins formed the Sc A and Sc B group. However, the twelve different subgroups obtained in this study suggests possible and frequent occurrence of mutants in *S. aureus* in different host cells. Historically, *S. aureus* has been described as a variable bacterium with many pathogenic and antibiotic resistance variants (Coltman, 1979; Kloos and Schleifer, 1981). The limited number of morphological and

cultural characters of *S. aureus*, and the lack of standardization of cultural conditions and virulence test among different researchers have led to confusion and uncertainty in the characterization of this pathogen (Kloos and Schleifer, 1981). Distinct phenotypes usually consist of isolates that are genetically less related and such identification of isolates using biochemical, cultural and morphological techniques often lack consistency and precision (Kloos and Schleifer, 1981). In the current study, we have found that identification of genetic diversity in *S. aureus* depends on sources of isolates, different host cells and occurrence of mutants. For instance, two isolates genotyped as Sc C were originated from human while four and six isolates respectively from plant and animal origins were genotyped as Sc A and Sc B (Figure 3). Besides, the possible and frequent occurrence of mutants in *S. aureus* constitutes the broad genetic variation that exists within Sc A, B and C genotypes.

RAPD markers revealed possible relationship between host origin, mutation and genetic variation among *S. aureus* isolates, and this demonstrated its fingerprinting and diagnostic potential. Obviously, for these DNA bands patterns to have a practical meaning in the areas of medicine, population biology and epidemiology, specific DNA bands must be related to host origins, mutation and virulence genes (Welsh and McClelland, 1990). This could be accomplished by a systematic comparison of DNA band patterns among bacteria contrasting for the different host origins, mutation and virulence genes present. Similar approach has been used to differentiate aggressive from non-aggressive isolates of the oilseed rape pathogen *Phoma lingam* (Schafer and Wostmeyer, 1992).

The DNA fingerprint defined for each race of *S. aureus* should be useful for epidemiological surveys, medical diagnoses, and in the identification of new virulent strains and isolates and their origin.

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