Molecular identification of MRSA Stains of Clinical Staphylococcus aureus Isolates which produce Enterotoxin using ISSR markers

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Abstract

Staphylococcus aureus were isolated from clinical cases of human skin infections and subjected to a molecular study. Genomic DNA extracted Commercial kit method and the yield was observed; it ranged from 240-490 ng/ml. This method gave an A260/A280 ratio approximately 1.7 of DNA quality. This research approaches to investigate the genetic diversity of the methicillin resistant Staphylococcus aureus strains that are clinically collected by performing ISSR PCR technique The aim of this study to isolate and identified of S.aureus from different gastrointestinal infection and wound sources and study the genetic diversity among these isolates by using ISSR markers. Collected 20 samples from 5samples were isolated S.aureus isolates were characterized depending on its morphological and biochemichal , then extracted DNA from its. ISSR primers has great a binding to production unique band, when 9primers from 10primers, ISSR9 was produce (5) unique bands, while The dendrogram of issr was reverted than isolates number 2 and 3 had the great genetic diversity 0.0.987 while the isolates number 5 and 4 had the lowest genetic similarity 0.065 in contrast with other isolate have great genetic diversity .

Introduction

The staphylococcal enterotoxins are recognized agents of intoxication staphylococcal food poisoning syndrome and may be involved in other types of infections with sequelae of shock in humans and animals. Serologically, five toxin groups have been recognized and designated staphylococcal enterotoxin A (SEA), SEB, SEC, SED, and SEE. Minor epitope differences in the SEC group have resulted in a further subdivision into SEC1, SEC2, and SEC3. SEA, SED, and SEE share immunological determinants, as do SEB and SEC1 and streptococcal pyrogenic exotoxin A (SPEA) [1].

Staphylococcal enterotoxins (SEs) are exoproteins which, when ingested, induce gastro-enteric syndrome in humans and can cause toxic shock. Staphylococcal food poisoning outbreaks are characterized by vomiting and diarrhea and occur quite

frequently worldwide; hence, the detection of enterotoxins is epidemiologically essential [2]. The SEs are emetic toxins and are classified as members of the pyrogenic toxin superantigen family because of their biological activities and structural relatedness [3]. These proteins (MW: 35 kDa) are not inactivated by heating to 100℃ for 15–30 minutes (Kayser et al., 2005 p232). [4,5]. In recent years, different molecular typing techniques have been applied to study the genetic diversity of s.aureus and the possible occurrence of similarity and difference between them, (ISSR) analysis can be performed as a method for study genetic diversity with large number of different strains of microorganisms. It is inexpensive and requires less amount of DNA [6]. Moreover, ISSR analysis is technically being commonly used as an indicator for determination the genetic diversity, based on variation found in the regions between microsatellites it has been used in genetic fingerprinting gene tagging and detection of clonal variation [7]. This technique which involved amplification of DNA segment present in between two identical microsatellite repeat regions by addition the oriental in opposite direction with suitable distances ISSR method has been reported produce more complex markers patterns .In addition, ISSR method are more reproducible method Because ISSR primers designed to anneal temperature to a microsatellite sequences, allowing higher annealing temperature to be used. It also because of multilocus fingerprinting profile obtained ISSR has been found to be an efficient, low cost, simple operation, high stability and abundance of [8].The aim of the study Detection of the unique bands and polymorphism between S.aureus isolates.

Materials and Methods.

S.aureus isolates.

A total of 5 *Staphylococcus aureus* clinical isolates as shown in (table-1) were examined according to their microscopic features and biochemichal, and were sub cultured on nutrient Agar, for using in DNA extraction.

Genomic DNA extraction

The DNA was extracted by small-scale method commercial kit (Bionner-Korea) DNA Purity was measured depending on optical density by spectrophotometer. DNA quality was visualized by agars gel electrophoresis with ethidium bromide and visualized under UV light [9]

Molecular Analysis

ISSR assay

Six of ISSR primers were used they were provided by (Bioneer – Korea) in lyophilized form and dissolved in sterile distilled water to get final conc. of (10pmol/ml) [10]. Recommended by

provider the primers which tested in this study which list in (table-1)

Final concentration was performed in a volume of 25µl. PCR program for ISSR assay using the following program: No. of cycles= 40 cycles between initial denaturation and final extension, the following table shows the ISSR program:

Each PCR amplification reaction was repeated twice to ensure reproducibility the products analyzed by electrophoresis in 1.5% agars gels with stained ethidium bromide 0.5µl at 5vt/cm for 2hour.

Data analysis

Estimation of molecular weight

Computer software Photo-Capture M.W. program was used to determine molecular weight based on comparing the RAPD-PCR and ISSR-PCR products depending on molecular weight of bands and number bands of a 2000bp DNA ladder Bioneer (which consist of 13 bands from 100 to 2000 bp.)

Estimation of polymorphism, efficiency and discriminatory power

Data generated for molecular weight RAPD and ISSR markers result bands were a score for each bands on the molecular size (1 for present, 0 for absence) the commercial soft word [11]. Only major bands consistently amplified were scored. Polymorphism of each primer was calculated based on the following formula:

Polymorphism % = (Np / Nt) × 100

Where Np = the number of polymorphic bands of random primer, Nt = the total number of bands of the same primer. Efficiency and discriminatory power of each primer calculated according to the formula below:

- **Efficiency =number of polymorphic bands to each primer / total number of bands to the same primer.**
- **Discriminating power= number of polymorphic band to each primer / total number of polymorphic band to all primer X100 %.**

Primer efficiency ranged between (0-1). Discrimination power of each primer

Results and discussion

ISSR-PCR analysis

In this study, ISSR-PCR technique was used to reveal the genetic diversity among different studied *S.aureus* isolates in order to search the genetic diversity between *S.aureus* isolates and study the differences between them. A total of 178 use full bands were scored from the amplified products with the seven Inter Simple Sequence Repeat (ISSR), 120 bands were polymorphic, with average of (6) polymorphic bands ISSR10, and ISSR6

produce 32 polymorphic bands with average range size (100-2000)bp. (figure-1). Pr2 primers can be produce high unique bands can be produce 5 unique bands, (table-2)

From genetic distance, the ratio of genetic similarity among the five *S.aureus* isolates from 0.65791 to 0.06899, showing (table-3). The highest similarity 0.97868 (99.8%) was obtained between straien number (1 and 2) while 0.21556 (21.5%) genetic similarity between isolate number 3 and 2, but lowest level of similarity 0.1025 (10.2%) was appears between isolate number (3 and 4).

During dendrogram were constructed based on Nei and Lei (1979). Genetic distance using UPGMA cluster analysis and depicted genetic relationship among seven *S.aureus* isolates showing two major cluster, first cluster contained two main distance, these were introduced from patients sources and isolate number 2 formed separated line that came from another patient case, and second cluster contained isolate number with lowest genetic similarity 0.1025. These isolates introduced from patients wound sources, (figure-2) group, first group contain isolates number (1, 2) with higher genetic similarity 0.6782

In this study, each of genetic distance based on don't show geographic profiling between isolates. It has been reported that the dendrogram generated by ISSR better with genealogy and the know pedigree of the ISSR, on the another hand, Numbers of analysis studies used ISSR were found that ISSR produce more information with fewer number primer than number RAPD primer, during among study found a number polymorphic bands was still higher with less number [12,13]. ISSR less primers means less time, less DNA, less supplied and less samples, RAPD markers don't have the specific target comparing to ISSR markers. In fact ISSR markers are known to be more sensitive than RAPD markers, in this study it was obvious that the dendrogram based on ISSR markers was not in according with the dendrogram based on ISSR markers, thus, both dendrogram are in agreement with the groups of clinical origin the differences in clustering pattern of genotypes using ISSR markers also may be attributed to markers sampling error and the level of polymorphic detected[14].

Conclusion

ISSR markers produced high rate from polymorphism depending on polymorphic rate, the ISSR technique can be produce high level from unique bands a comparative with another markers, ISSR less efficiency in dendrogram results.

References

1. **Johnson** W. M., TyleR S. D., Ewan E. P., Ashton F. E., Pollard D. R., and Rozee K. R., Detection of Genes for Enterotoxins, Exfoliative Toxins, and Toxic Shock Syndrome Toxin 1 in Staphylococcus aureus by the Polymerase Chain Reaction, *J. of Clinic. Microbiol.* 1991, 29 (3):426.

2. **Tremaine** M. T., Brockman D. K. and Betley M. J., Staphylococcal Enterotoxin A Gene (sea) Expression Is Not Affected by the Accessory Gene Regulator (agr), *J. of Infect. And Immun.*, 1993, 61(1):356-359.

3. **Becker** K., Roth R. and Peters G., Rapid and Specific Detection of Toxigenic *Staphylococcus aureus*: Use of Two Multiplex PCR Enzyme Immunoassays for Amplification and Hybridization of Staphylococcal Enterotoxin Genes, Exfoliative Toxin Genes, and Toxic Shock Syndrome Toxin 1 Gene, *J. of Clin. Microbiol.* 1998, 36 (9): 2548-2553.

4. **Sharma** N. K., Rees C. E. D. and Dodd Ch. E. R., Development of a Single-Reaction Multiplex PCR Toxin Typing Assay for *Staphylococcus aureus* Strains, *J. of Appl. Environ. Microbiol*. 2000, 66 (4): 1347-1353.

5. **Naffa** R. G., Bdour S. M., Migdadi H. M. and Shehabi A. A., Enterotoxicity and genetic variation among clinical *Staphylococcus aureus* isolates in Jordan, *J. of Med. Microbiol.* 2006, 55:183-187.

6- Panepinto, J.C., Pardo, J.; Urban, C.; and Galvez, E.M. **2010**. The mitochondrial protein Bak is pivotal for gliotoxin-induced apoptosis and a critical host factor of *Aspergillus fumigatus* virulence in mice. *J. Cell Biol*; 174:509–519. .

7-Bornet, B. and Branchard, M. **2001**. Nonanchored Inter Simple Sequence Repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. *Plant Mol. Biol. Rep.***19**:209-215.

8- Zietkiewics, E.; Rafalski, A. and Labuda, D. **1994**. Genome fingerprinting by Simple Sequence Repeat (SSR) anchored polymerase chain reaction amplification. *Genomics.* **20**: 176-183.

9-Sambrook, J. and Russell, D. W. **2001**. Molecular Cloning. In: "A Laboratory Manual". Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

10-Hatti, A. D., Taware, S. D., Taware, A. S., Pangrikar, P. P., Chavan, A. M. and

Mukadam, D. S. **2010**. Genetic diversity of toxigenic and non-toxigenic

11-Bibi, S., Dahot, M. U., Khan, I. A., Khatri, A.,Naqvi, A. (2011). Study of genetic diversity in wheat (*Triticum aestivum L.*) using random amplified polymorphic DNA (RAPD) markers. *Pakistan J. Botany* **41**, 1023-1027.

12 - Tang Y-W. And Stratton C. W., Advanced Techniques in Diagnostic Microbiology, Springer, New York, USA, 2006.p 426,429.

13-Kareem N.H., Sabbah M. A., Ibraheem A. N., Said A.M., Development of SYBR Green Real time PCR for Identification Methicillin-Resistance *Staphylococcus aureus* (MRSA), *J. of Biotech. Research Center*, 2013, 7 (1): 67-71.

Table (1): *S.aureus* isolates examined during this study

S.aureus	Sources
S.aureus 1	Clinical
S.aureus 2	Clinical
S.aureus 3	Clinical
S.aureus 4	Clinical
S.aureus 5	Clinical

Table (1): The names and sequences of the primers used in this study

No.	Primers Name	Sequence(´5-´3)
1.	Pr1	GACAGACAGACAGACA
2.	Pr2	AGAGAGAGAGAGAGAGAGAGC
3.	Pr3	AGAGAGAGAGAGAGAGAGAGT
4.	Pr4	CTCTCTCTCTCTCTCTCTCTA
5.	Pr5	CTCTCTCTCTCTCTCTCTCTG
6.	Pr6	CTCTCTCTCTCTCTCTCTCTT

Following master amplification reaction

Materials	Final concentration	Volume for 1tube
PCR Premix	1x	5µI
Deionised D.W.	—	17 µI
Primer(10pmol/ml)	10pmol/ml	2µI
DNA template	100ng	1µI

Steps	Temperature(°C)	Time (min.)
Initial denaturation	94	5
Denaturation	94	1
Annealing	50	1
Extension	72	1
Final extension	72	10

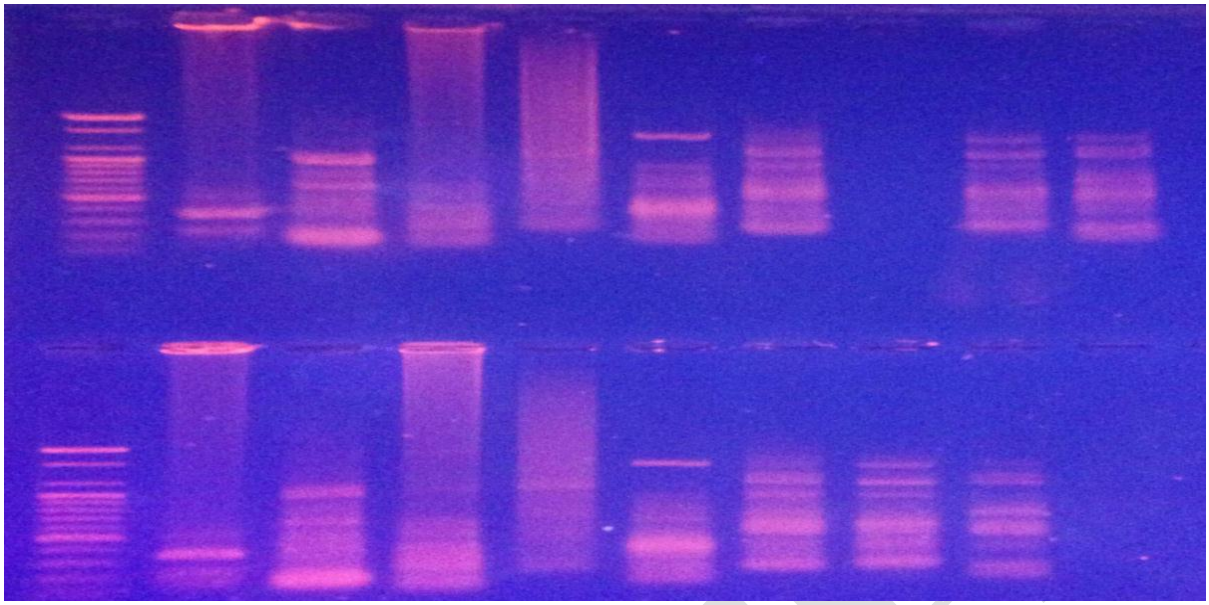

Table (2): Distinct characteristics of ISSR primers including in the study: primers name, total number of bands, number of polymorphic bands, number of unique bands, percentage of polymorphism, primer efficiency and discrimination value.

No.	Name of primers	Total number of main bands	Number unique bands	Number polymorphic bands	Polymorphism %	Primer efficiency	Discrimination power
1	P1	15	2	10	6.66	0.66	**66.6**
2	P2	16	0	6	6.25	0.37	**37.5**
3	P3	19	0	19	5.26	1	**100**
4	P4	9	0	4	11.1	0.44	**44.4**
5	P5	19	1	9	5.26	0.47	**47.3**
6	P6	22	1	17	4.54	0.77	**77.2**

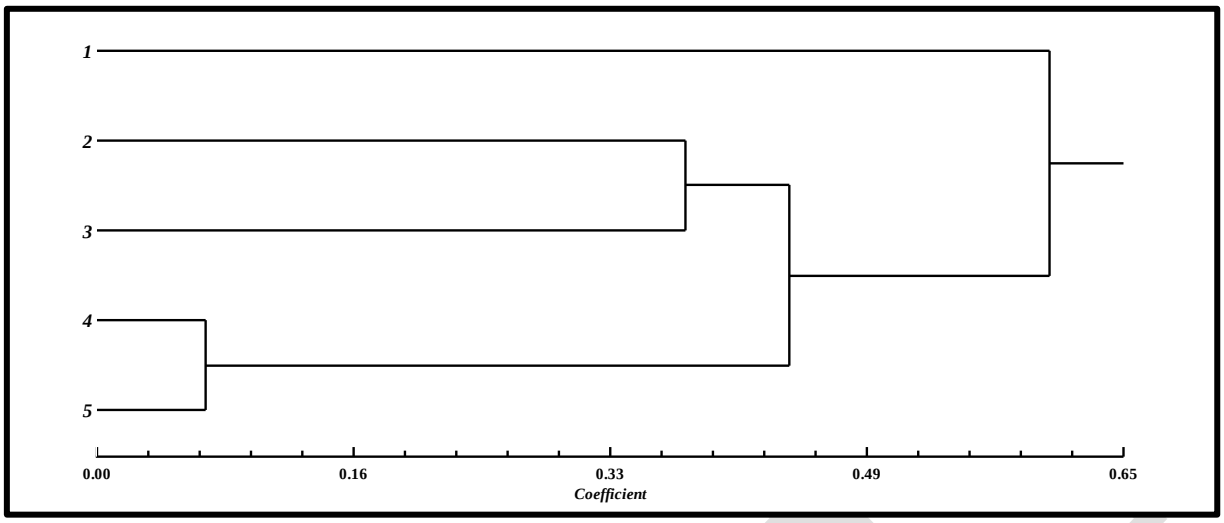

Table (3): Values of genetic distance among *S.aureus* Isolates calculated according to Nei and Lei, 1979).

	1	2	3	4	5
1	0.00000				
2	0.51285	0.00000			
3	0.65791	0.41926	0.00000		
4	0.86553	0.4055	0.51083	0.0000	
5	58380	0.41926	0.40547	0.06899	0.0000