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Alyaa Mohammed H

Maysaa Adil Ali

Mohammed Saad Alnasari

Wedean Al-Hadban

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Application of Randomly Amplified Polymorphic DNA (RAPD) Technique to Estimate Genetic Distance among Some Methicillin Resistant *Staphylococcus aureus* Isolated from Different Iraqi Hospitals

***Alyaa Mohammed H.*[1*]** ***Maysaa Adil Ali*[2]** ***Mohammed Saad Alnasari*[3]**
***Wedean Al-Hadban*[3]**

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Abstract:

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the principal nosocomial causative agents. This bacterium has the capability to resist wide range of antibiotics and it is responsible for many diseases like skin, nose and wounds infection. In this study, randomly amplified polymorphic DNA (RAPD)-PCR was applied with ten random primers to examine the molecular diversity among methicillin resistant *Staphylococcus aureus* (MRSA) isolates in the hospitals and to investigate the genetic distance between them. 90 Isolates were collected from clinical specimens from Iraqi hospitals for a total of 90 isolates. Only 10 strains (11.11%) were found to be MRSA. From these 10 primers, only 9 gave clear amplification products. 91 fragment lines were generated from these primers across all isolates with an average of 10 fragment lines per primer. Of these, 90 (99%) were polymorphic. The size of the amplified bands ranged between 145-2109 bp. The polymorphism percentage for all primers was 100% except OP-X17 primer which gave 86% polymorphism. The genetic distances revealed from Jaccard similarity index was calculated for the 90 RAPD polymorphic fragment lines. The highest genetic distance value 0.959 was between isolate number (1) and (5) and between isolate number (3) and (10), while the lowest genetic distance value 0.218 was between isolate number (6) and (7). This study shows that RAPD-PCR technique assayed with nine primers can be successfully applied to reveal the genetic distances among methicillin resistant *Staphylococcus aureus* (MRSA) isolates from different hospitals.

Keywords: Methicillin resistant *Staphylococcus aureus* (MRSA), randomly amplified polymorphic DNA, Genetic distance, DNA typing.

Introduction:

Staphylococcus aureus is an opportunistic pathogen that causes nosocomial infections in both developing and developed countries. Some strains are also responsible for human food poisoning as they produce enterotoxins in food stuffs (1, 2, 3). Although immense advances in medical care, Methicillin-resistance *S. aureus* (MRSA) is still responsible for high mortality rate as a pathogenic agent in both hospital and community environment. MRSA was first reported in 1961 and has become an emerging pathogen in both hospitals and intensive care units as well as in community settings especially in congested places (4, 5). Presently, these bacteria have developed their resistance against different types of antibiotics and it considered the main causative agent for hospital acquired infections around the world (6, 7).

The idea of Methicillin resistance for these bacteria depends on SCCmec which is Staphylococcus Cassette-Chromosome (SCCmec). The natural resistance of *S.aureus* towards methicillin is caused by the expression of mecA gene, a gene produce protein called penicillin binding protein 2a (8, 9). To control the infection of *S. aureus*, precise and rapid typing is required and several techniques have been used in other studies (10, 11).

Today, there are numerous conventional methods available such as bacteriophage typing, ribotyping and capsular typing to verify the genetic variability along with the patients isolates (12). Such methods considered as difficult and required a long preparation. However, the PCR-based techniques are fast and easy to perform and require limited specialized equipment. Randomly

[1]Department of Pathological Analyzes, Institute of Medical Technology/ Al- Mansour, Baghdad, Iraq.
[2]Department of Applied Sciences, University of Technology, Baghdad, Iraq.
[3] Biotechnology Department, College of Sciences, University of Baghdad, Baghdad, Iraq.
Corresponding author: alyaa_mhadi@yahoo.com

Amplified Polymorphic DNA (RAPD), a simple PCR based method, is used widely for epidemiological studies. RAPD primers can efficiently scan whole chromosomes for the presence of short inverted DNA sequences and amplifies the inverted fragments. The difference in the length of amplification products can be used to differentiate the genetic diversity and to construct genetic fingerprints. This technique needs a low concentration of DNA using short synthetic oligonucleotide primers in length. Depending on their versatility and easy handling, RAPD-PCR technique is extensively used in epidemiological study of MRSA. Moreover, it is very useful for obtaining an accurate microbial database linking genetic marker and their clinical outcomes in order to control their spread (13). The aims of this study are (i) investigate the genetic diversity for MRSA strains obtained from different Iraqi patients using RAPD-PCR, and (ii), the assessment of genetic distance among each isolates using RAPD-PCR technique.

Materials and Methods:

Isolation and Identification of MRSA

All 10 strains of *Staphylococcus aureus* were isolated from Iraqi patients attending different hospitals in Baghdad city. Isolates were obtained from nasal, blood and wound samples. *Staphylococcus aureus* isolates were identified by their phenotypic characterizations in different biochemical tests including oxidase, coagulase, and catalase (14). For genotypic identification, the obtained *Staphylococcus aureus* isolates were detected for the presence of mecA and nuc genes by polymerase chain reaction and all were confirmed to be MRSA isolates.

DNA preparation

The genetic material of the 10 MRSA isolates was isolated via the Promega DNA extraction kit, with 30 µg/ml lysozyme enzyme. Bacterial colonies were grown overnight in brain heart infusion broth at 37 ºC. 1 ml of overnight bacterial growth was centrifuged at 10000 rpm for 5 min. All the extraction steps were followed and the provided solutions were added according to the manufacturer´s recommendations with one additional step which is the 1-hour treatment of bacterial cells with lysozyme prior to extraction steps. Spectrophotometer was used to assess both concentration and purity of the extracted DNA samples, and 0.8% agarose gel was used for checking DNA integrity using gel electrophoresis unit., Subsequently, DNA bands were examined under UV light after staining with ethidium bromide (15).

Primer selection and RAPD- PCR analysis

In this study, 10 random primers from (Alpha DNA, USA/Canada) were examined. All primers produced results regarding to amplification and polymorphism including (OP-D18, OP-D20, OP-T07, OP-W02, OP-X12, OP-X17, OP-A03, OP-X06 and OP-Y13) except (OP-A06) which gave no amplified products (Table.1). Amplification was performed using thermal cycler (Labnet international. Inc - USA). A volume of 25 µl was used as a PCR reaction volume mixture including 12.5 µl (1X) Green Master Mix (Promega-USA) consisting of 10mM Tris-HCl (PH8), 50mM KCL, 1.5mM MgCl2, 200µM each deoxynucleotide triphosphate (dNTP) and 1U DNA polymerase. Thermocycling conditions were set at 94°C for 5 min as initial denaturation, and 45 cycles of 1 min at 94°C, 36°C for 1 min and 72°C for 2 min, and a final extension step at 72°C for 10 min, followed by a hold step at 4°C (16, 17). Each DNA sample was amplified twice using the same PCR conditions with the elected primer to ensure the accurate result. Twenty microliters of PCR amplicons were fractionated by electrophoresis in 2% agarose gel at a constant voltage of 5 volt/cm for 2 hour using 0.5X concentration of TBE buffer, which consisted of 10mM Tris-Borate and 1 mM EDTA. It was compared along with 100bp DNA ladder (Promega-USA). The DNA bands were analyzed using UV transilluminator after staining with ethidium bromide.

Table 1. The sequences of the RAPD primers.

No.	Primer name	Sequense '5--------3'
1	Op - D18	GAGAGCCAAC
2	Op - D20	ACCCGGTCAC
3	Op - T07	GGCAGGCTGT
4	Op - W02	ACCCCGCCAA
5	Op - X06	ACGCCAGAGG
6	Op - X17	GACACGGACC
7	Op - X12	TCGCCAGCCA
8	Op - Y13	CACAGCGACA
9	Op - A03	AGT CAG CCAC
10	Op - A06	GGT CCC TGAC

Data analysis:

Molecular weight estimation

Molecular weight for the DNA bands generated from each primer was assessed by using Photo-Capture M.W. program version 1.0 (18). A 100 bp ladder was run together with PCR products as a molecular weight marker.

Primers parameters

Overall bands numbers were scored visually. Both polymorphic fragments and fragment lines were also scored. Polymorphism percentage, discrimination power and efficiency for each

studied primer were determined using the following formula:

Polymorphism percentage= No. of polymorphic fragments\ No. of fragment lines amplified by the same primer.

Discrimination power= No. of polymorphic fragments\ total No. of polymorphic fragments obtained.

Efficiency= total No. of bands amplified by primer\ total No. of bands gained.

All scorable bands were transformed into a binary (0-1) matrix ("1" indicate presence and "0" indicate absence) (19).

Genetic distance and Eigenvalue analysis

The polymorphic fragments obtained from RAPD primers in this study were analyzed. The best clean and reproducible amplified bands had been taken into consideration for this study. Genetic distance among the 10 isolates was estimated. Eigenvalue and variance for each isolates were also assessed. All computations were carried out using Palaeontological Statistics (PAST) Software version 1.62 (20).

Results and Discussion:

DNA amplification

Genetic distances among ten MRSA isolates were analyzed using ten different RAPD primers. The ability for each primer for estimation diversity among MRSA isolates was varied. Among these ten decamer primers, there was one primer (OP-A06) that failed to generate PCR products with all MRSA isolates, therefore, it was eliminated from the analysis. The remaining nine primers showed reliable banding patterns.

RAPD-PCR and Primers parameters

The RAPD-PCR technique is considered one of the most significantly used DNA typing methods. In earlier studies, the distribution of *S. aureus* and its root of transmission have been investigated using this technique with several RAPD oligonucleotide (21). The RAPD primers used in this study generated 263 total bands with an average of 29 bands per primer (Table. 2). Each isolate was varied in term of the generated bands, Primer OP-X06 produced 4 bands showing the lowest primer efficiency (1.52%), while primer OP-D18 produced 53 bands showing the highest primer efficiency (20.15%). The molecular weight of the amplified bands was ranged from 145 bp (OP-T07) to 2109 bp (OP-D18). RAPD primers amplified 91 fragment lines across all isolates genomes with average of 10 fragment lines per primer. Among these fragment lines scored, 90 fragment lines (99.0%) were polymorphic with average of 10 polymorphic fragment lines per primer across the 10 MRSA isolates. Primer (OP-X12) amplified 15 fragment lines (100% polymorphism) representing the maximum discrimination power (17%), while primer OP-X06 amplified 2 polymorphic fragment lines (100% polymorphism) representing the minimum discrimination power (2.2 %).

Table 2. RAPD primers parameters.

Primer name	Sequence (5 - 3)	Total bands (No.)	% primer efficiency	No. of fragment lines	Polymorphic fragments (No.)	% polymorphism	% discrimination power
OP-A03	AGTCAGCCAC	52	19.77	14	14	100	15.5
OP-D18	GAGAGCCAAC	53	20.15	13	13	100	14.4
OP-D20	ACCCGGTCAC	24	5.32	9	9	100	10
OP-T07	GGCAGGCTGT	30	11.40	12	12	100	13.3
OP-W02	ACCCCGCCAA	33	12.54	13	13	100	14.4
OP-X06	ACGCCAGAGG	4	1.52	2	2	100	2.2
OP-X12	TCGCCAGCCA	27	10.26	15	15	100	17
OP-X17	GACACGGACC	22	8.36	7	6	86	7
OP-Y13	GGGTCTCGGT	18	6.84	6	6	100	7
Total		263		91	90		
Average		29		10	10		

The arbitrary primers (OP-X12, OP-A03, OP-D18 and OP-T07) were useful for discrimination MRSA isolates of distinct characteristics (Fig. 1 and 2). 100% polymorphism percentage was recognized for all primers except (OP-X17) primer among the studied isolates. This considered high level percentage of polymorphism produced by random primers when it is compared to other RAPD studies in MRSA which were 93.9% (22) and 88.24% (3).

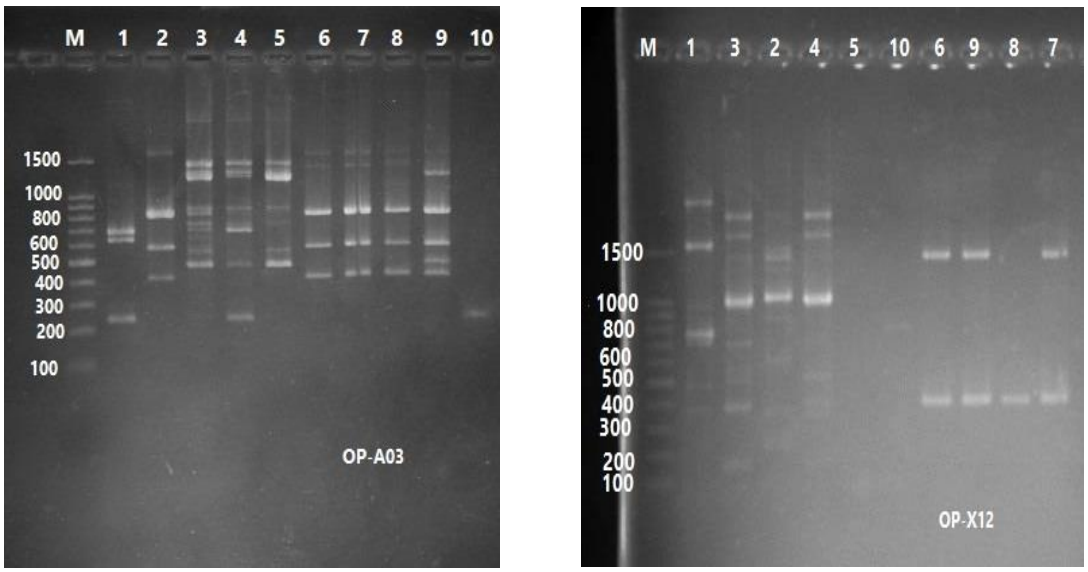

Figure 1. Agarose gel electrophoresis of primer OP-X12 and OP-A03 for DNA samples of the MRSA isolates. M: represent 100 bp ladder. Lanes: from 1-10 represent MRSA isolates.

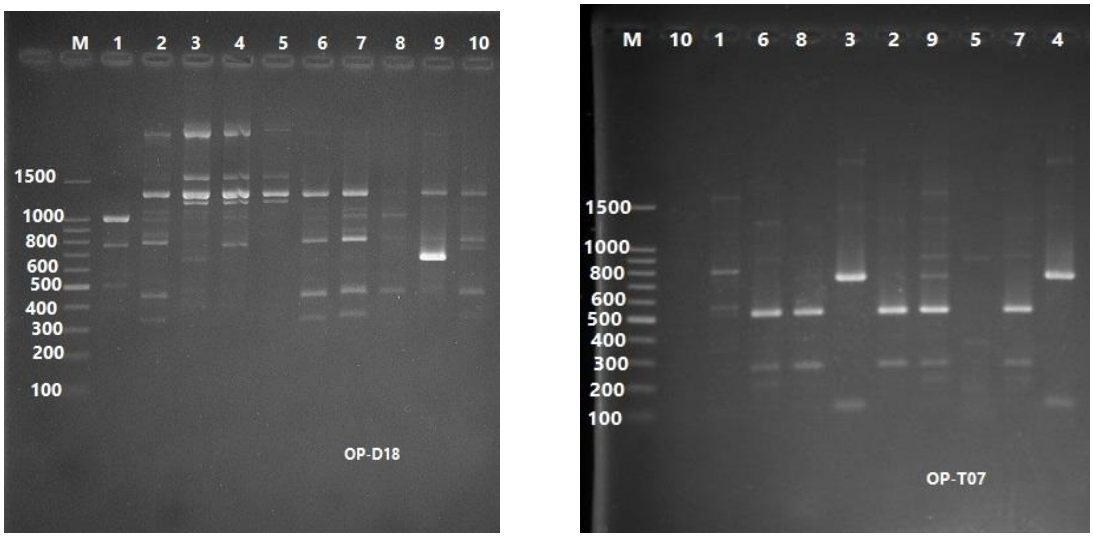

Figure 2. Agarose gel electrophoresis of primer OP-D18 and OP-T07 for DNA samples of the MRSA isolates. M: represent 100 bp ladder. Lanes: from 1-10 represent MRSA isolates.

Genetic distance and Eigenvalue estimation

The genetic distances (GD) revealed from Jaccard similarity index was calculated for the 90 RAPD polymorphic fragments according to formula (GD = 1 - similarity) (23), of the 10 MRSA isolates using PAST software version 1.62. Table 3 illustrates the genetic distance values among MRSA isolates. The genetic distance values ranged from (0.218) to (0.959). The isolate number 1 was highly divergent from isolate number 5 as well as isolate number 3 from isolate number 10 with a distance of 0.959. Isolate number 6 was very closely related to isolate number 7 with distance of 0.218. Eigenvalue and the variances for the studied isolates were also estimated using PAST software version 1.62, as seen in Table 4. Eigenvalue represents the amount of dispersion of samples from the mean. From the results obtained, it is obvious that there is a positive correlation between variance and Eigenvalue. Whenever the variance value increases, the Eigenvalue will also increases (Fig.3). According to the results obtained, the maximum result of genetic distance was high in parallel to the report of other studies in which scored a 0.507 genetic distance, while the minimum result of genetic distance was comparatively high in parallel with results that showed a 0.085 genetic distance among a twenty three MRSA isolates in Pakistan (22).

Table 3. Genetic distance's values among MRSA isolates as revealed by RAPD analysis.

Isolates	1	2	3	4	5	6	7	8	9	10
1	0									
2	0.8864	0								
3	0.8966	0.8594	0							
4	0.8628	0.8449	0.3266	0						
5	**0.9592**	0.9091	0.4694	0.532	0					
6	0.8889	0.5953	0.8254	0.8071	0.8704	0				
7	0.907	0.4865	0.8549	0.8393	0.9057	**0.2188**	0			
8	0.871	0.625	0.9273	0.9184	0.9303	0.5938	0.5	0		
9	0.8948	0.6924	0.8422	0.868	0.8479	0.6667	0.6	0.577	0	
10	0.875	0.875	**0.9592**	0.9025	0.9445	0.8438	0.8667	0.8422	0.8847	0

Table 4. Variance values comparison among MRSA isolates revealed by RAPD analysis.

PC	Eigenvalue	% variance
1	7.04874	40.849
2	2.99164	17.337
3	1.84128	10.671
4	1.48272	8.5927
5	1.26591	7.3362
6	0.992022	5.749
7	0.702378	4.0704
8	0.626952	3.6333
9	0.303925	1.7613

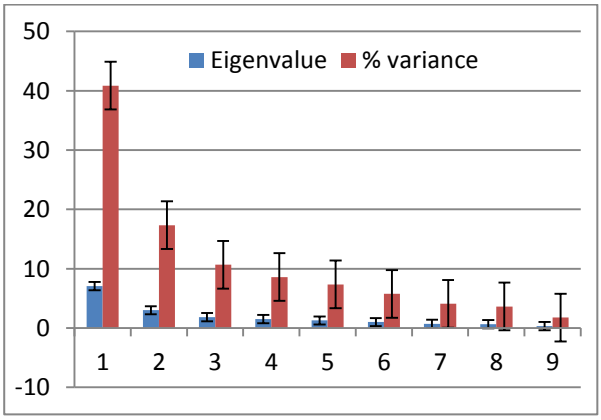

Figure 3. Variance values among the studied MRSA isolates with comparison with Eigenvalue.

Conclusion:

MRSA, with its virality, distribution and its ability to resist a wide spectrum of antibiotics, make this bacterium one of the most threating agents worldwide. According to the data obtained from this study, RAPD-PCR technique is considered a successful method for MRSA typing and to estimate genetic distances among the studied isolates with its simplicity, rapid and cost-efficient. It was confirmed that there was an extensive genotypic diversity of MRSA from various clinical samples using this technique. Consequently, in light of the data observed in this research and the DNA typing of MRSA isolates, this method can be used to monitor and understand the pathogenesis and the spread of this bacterium in hospitals, communities and moreover for helping the establishment of a successful surveillance procedures and facilitate global MRSA control.

Conflicts of Interest: None.

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تطبيق تقانة مؤشرات التضاعف العشوائي المتعدد الاشكال لسلسلة الدنا لتقدير البعد الوراثي بين بعض عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين والمعزولة من مستشفيات عراقية مختلفة

علياء محمد هادي[1] ميساء عادل علي[2] محمد سعد النصاري[3] وديان الهدبان[3]

[1] قسم التحليلات المرضية، المعهد الطبي التقني/ المنصور، الجامعة التقنية الوسطى، بغداد، العراق.
قسم العلوم التطبيقية، فرع التقنيات الاحيائية، الجامعة التكنولوجية، بغداد، العراق.[2]
[3] قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق.

الخلاصة:

تعتبر سلالات المكورات العنقودية الذهبية المقاومة للميثيسيلين واحدة من المسببات الرئيسية للإصابات الناتجة عن المستشفيات. هذه البكتيريا لها القدرة على مقاومة مدى واسع من المضادات الحيوية كما انها مسؤولة عن العديد من الامراض مثل اصابات الجلد، الانف والجروح. في هذه الدراسة تم استخدام تقانة مؤشرات التضاعف العشوائي لسلسلة DNA (RAPD) مع عشرة من البادئات العشوائية لفحص التنوع الجزيئي بين عزلات المكورات العنقودية الذهبية المقاومة للمثيسيلين وللتحقق من البعد الوراثي بينهم. تم جمع (90) عزلة من عينات سريرية ومن مستشفيات عراقية مختلفة ، ووجد أن (10) سلالات فقط وبنسبة (11.11٪) كانت مقاومة للميثيسيلين. من هذه البادئات العشرة، تسعة فقط قد اعطت نواتج تضاعف واضحة. انتجت هذه البادئات 91 حزمة رئيسية لجميع العزلات وبمعدل (10) حزم رئيسية لكل باديء، كانت من ضمنها (90) حزمة متباينة وبنسبة (99%). تراوحت احجام الحزم المضاعفة ما بين (145-2109) زوج قاعدي. النسبة المئوية للتباين الوراثي لكل البادئات كانت بنسبة (100%) ماعدا الباديء OP-X17 الذي اعطى نسبة تباين (86%). تم حساب الابعاد الوراثية بالاعتماد على مؤشر (Jaccard similarity) ل (90) حزمة رئيسية متباينة. اعلى قيمة بعد وراثي (0.959) كانت بين العزلة رقم (1) والعزلة رقم (5) وما بين العزلة رقم (3) والعزلة رقم (10)، بينما كانت ادنى قيمة بعد وراثي (0.218) بين العزلة رقم (6) والعزلة رقم (7). هذه الدراسة تظهر ان تقانة مؤشرات التضاعف العشوائي لسلسلة DNA والتي تم اختبارها مع 9 بادئات يمكن تطبيقها بنجاح للكشف عن الابعاد الوراثية بين عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين ومن مستشفيات مختلفة.

الكلمات المفتاحية: المكورات العنقودية الذهبية المقاومة للميثيسيلين، مؤشرات التضاعف العشوائي المتعدد الأشكال لسلسلة DNA، البعد الوراثي، تنميط DNA.