# Phylogenetic Analysis of Antibiotic Producing Bacteria Isolated from Soil Contaminated by Human Wastes using Random Amplified Polymorphic DNA (RAPD) Technique

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**Abstract:**

Antibiotic is one of the most important commercially exploited secondary metabolites produced by various microorganisms (as a bacterium or a fungus). The growing recognition of the pressing need for new antimicrobial agents for the treatment of infectious diseases demands discovering new antibiotic-producing strains of bacteria.

In the present investigation, soil samples contaminated by human feces were collected from different geographical regions belonging to Cairo, Assiut and Qena cities , Egypt; to isolate some strains of antibiotic- producing bacteria and characterize the isolates using RAPD- PCR technique. One hundred and ten strains of bacterial isolates were isolated from the collected soil samples. From which five selected isolates (D110, D3, D103, D106 and D104) showed promising results for an effective antibiotic production according to the microbial sensitivity tests with two Gram positive and two Gram negative bacterial strains by forming a clear zone of inhibition. The results showed that the average of clear zones of Gram positive bacteria are wider than the average of clear zones of gram negative. Such isolates have more ability to inhibit the growth of Gram positive bacteria than to inhibit the growth of Gram negative bacteria. The five coded selected strains were identified as, *Bacillus licheniformis, Bacillus pumilus, Staphylococcus epidermidis, Serratia marcescens* and *Paenibacillus lautus* , respectively.

In addition, it has been possible to differentiate between the isolated strains at the molecular level using RAPD-PCR. The results showed that these isolates can be described according to the banding- profile pattern. The five selected isolates were grouped in four clusters; based on genetic similarity. The first and the second groups were represented by D106 and D103 respectively. The third group included the two isolates; D104 and 110 and the strain D3 represented the fourth group.

**Keywords**: Phylogenetic , antibiotic , human wastes , RAPD

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**Introduction:**

Antibiotics are one of the most important commercially exploited secondary metabolites produced by bacteria. The remarkable increase in antibiotics resistant bacterial species (Motta *et al*, 2004; and Huda Sheikh , 2010 ) lead to search for new sources of antibiotics through the isolation and identification of new types of microorganisms such as bacteria, fungi and actinomycetes (Alexander, 1982). Bacillus species producing antibiotics have been used as biocontrol agents against pathogenic fungi (Yilmaz, *et al.*, 2005). More than 1200 isolates of lactic acid bacteria isolated from different environments were screened for antifungal activity, from which approximately 10% represented inhibitory activity and 4% showed strong activity against *Aspergillus fummigatus* (Magnusson, *et al*., 2003).

Soil is considered one of the most suitable environments for microbial growth (Cavalcanti, *et al*., 2006). Most of the antibiotic producers used today are soil microbes. The genus *Streptomyces* , an antibiotics producer , has been isolated from the soil of Yemen (Ahmed, 2003). One hundred bacterial isolates were isolated from six different soil samples collected from Egypt (Gebreel, *et al*., 2008). They reported that twenty of them could antagonized some selected plants and human pathogenic fungi with varying degrees. Moreover, among twenty bacterial strains isolated from soil stressed agro-ecological niches of Eastern Uttar Pradesh, India, eleven isolates showed strong antimicrobial activities (Singh, *et al*., 2009). Fecal wastes from domestic, wild life animals and human applied to the soil may contain a wide variety of pathogenic viruses, bacteria, and parasites.

The traditional typing methods for discriminating different bacterial isolates of the same species are essential epidemiological tools in infection prevention and control. Traditional typing systems based on phenotypes, such as serotype, biotype, or antibiogram, have been used for many years. However, the developments of molecular methods have revolutionized our ability to differentiate among bacterial isolates (Sabat, *et al*., 2013). Random Amplified Polymorphic DNA - PCR (RAPD-PCR), which has been described by Williams, *et al*., (1990) is the most frequently used PCR- based techniques . In this techniques single or a pairwise combination of primers, typically 10 nucleotides in length, are used to amplify target sites for the primer happen to occur within approximately 5 Kb of each other on opposite DNA strand.

The present investigation aimed to isolate some strains of antibiotic-producing bacteria from soil samples contaminated by human feces collected from different geographical regions in Egypt and characterizing the isolates using RAPD- PCR technique.

**Materials and Methods:**

- **Collection of Soil samples:**

Five soil samples were collected from different locations belonging to Cairo, Assiut and Qena cities, Egypt. Soil samples were taken in sterilized polyethylene bag using sterilized spatula then transferred to the laboratory at the Department of Genetics, Assiut University. Samples were dried at air oven and then cooled to room temperature.

- **Isolation of bacterial isolates:**

Suspensions were made by adding 1g of each soil sample to 100 ml sterile saline solution. Ten fold dilutions of these suspensions were planted on Luria – Bertani (LB) agar medium and incubated at 37°C for 72 hours. Single colonies of the grown bacteria which varied in shape and color were picked up using a sterile loop and purified by streaking on nutrient LB agar. The bacterial isolates were kept on LB slants agar and stored at 4° C and recultured monthly.

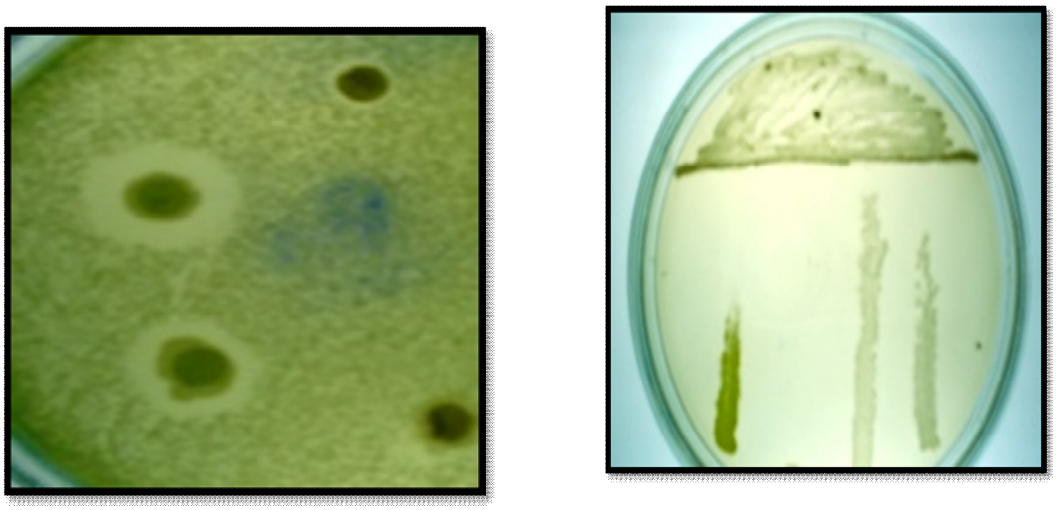
- **Testing pure isolated cultures for their abilities to produce antibiotics:**

The bacterial tester strains, which were used to detect antibiotic activity of isolated strains, were delivered from Medical Microbiology and Immunity Department, Faculty of Medicine and, Dairy Science Department, Faculty of Agriculture, Assiut University, Assiut, Egypt. The tester strains were chosen according to (Narisawa, *et al*., 2008; and Chandrashekhara, 2010) and included the following different genus of bacteria:

I) Two Gram positive Bacteria: *Bacillus subtilis* and *Staphylococcus aureus.*

II) Two Gram negative Bacteria: *Escherichia coli* and *Pseudomonas aeruginosa*.

All isolates were tested for antibiotic production by two different methods: Filter disk assay (Fitri and Bustam, 2010) and Agar Streak Method (Basavaraj, *et al*., 2010; and Chandrashekhara, 2010). Fig (1) shows an example of antibiotic activity of isolated strains tested by the two methods.



**(a)** **(b)**

**Fig (1)** An example of antibiotic activity of isolated strains tested by:

a- Filter disk assay

b- Agar Streak

The selected isolates were characterized morphologically. The morphological features were colony form, surface, margin, elevation, and colour in addition to cell shape and gram stain.

• **Extraction of genomic DNA for PCR:**

The extraction of total bacterial genomic DNA was performed according to procedures described by Sambrook *et al*. (1989) with some modifications. Briefly, Cells from 50-ml cultures were harvested by centrifugation and the pellets were resuspended in 2 ml TEN buffer [150 mM NaCl, 10 mM Tris-HCl , 100 (pH= 8.0), 100 mM EDTA (pH=8.0) containing 4 mg/.mL lysozyme. The cells were grounded in liquid nitrogen then, 800 µl of 60ºC CTAB extraction buffer (100mM Tris-HCl, 1.4M NaCl, 20mM EDTA, 2% hexadecyltri-methyl-ammonium bromide) was added , adjusted to pH=8.0 and incubated at 60 °C for 30 min. The DNA was extracted by adding 1 volume of chloroform-isoamyl alcohol (v/v = 24:1, pH=8.0), and the mixture was mixed gently and centrifuged at 5000 rpm for 20 minutes at 10ºC. Then the aqueous phase was transferred to clean tube and the nucleic acids were precipitated by adding 0.1 volume of sodium acetate (pH=5.2) and 0.6 volume of isopropanol to the supernatant. The mixture was incubated in ice for 30 min and centrifuged (10,000 rpm for 5 min at 4 °C) to recover the precipitated nucleic acids. The pellets were washed with 70% ice-cold ethanol and centrifuged at10, 000 rpm for 2 min at 4 °C). The pellets were dissolved in 300-500 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8·0) and treated with 3µl RNAase and incubated in 37ºC for 30 minutes. Samples were then treated with 3µl proteinase-K and incubated at 37ºC for 30 minutes. The DNA was precipitated, washed, and dissolved in TE as above. The DNA samples were kept at -20°C.

• **RAPD-PCR and electrophortic analysis:**

Polymerase chain reaction (PCR) was conducted using ten arbitrary 10-mer oligonucleotied primers obtained from commercial sources (Operon Technologies, Alameda, CA. USA),Table (1) gives the codes and sequences of used Primers.. PCR reactions were conducted according to Williams *et al*. (1990). The reaction conditions were optimized and mixtures (25 µl total volume) were composed of 11.7 µl $dH_2O$, 3.0 µl 10X reaction buffer, 3.0 µl dNTP's mix, 2.0 µl primer, 4.0 µl $MgCl_{2,\ 0.3}$ µl Taq DNA polymerase and 1 µl Template DNA. The reaction was overlaid with mineral oil and thermal cycling achieved in TECHNE thermocycler (Model FTGEN5D, TECHNE, Cambridge Ltd, Duxford, and Cambridge, U.K.) according to the following program: initial denaturation at 85ºC for 3 min, followed by 40 cycles of 85ºC for 1 min, 33ºC for 2 min, 72ºC for 2 min, and a final extension at 72ºC for 10 min and subsequently cooled to 4ºC. PCR products were resolved by loading 14 µl of each reaction onto a 1.4% ultra pure agarose gel containing 0.01% ethidium bromide in 1X concentration Tris- acetate (TAE) buffer (89mM Tris-borate; 2.5mM EDTA). To visualize RAPD bands, electrophoresis was carried out under constant voltage of around 60V for approximately 3-3.5 hours. The patterns were visualized on a Transilluminator (Ultra-Violet Product, Upland, CA, USA,).

**Table (1): Codes and sequences of used Primers.**

| Serial No. | Primer codes | Sequence (5' to 3') |
|---|---|---|
| **1** | OPA01 | 5'-CAGGCCCTTC-3' |
| **2** | OPA08 | 5'-GTGACGTAGG-3' |
| **3** | OPA09 | 5'-GGGTAACGCC-3' |
| **4** | OPI09 | 5'-GAGTCAGCAG-3' |
| **5** | UBC01 | 5'-CCTGGGCTTC-3' |

**Analysis of RAPD banding patterns:**

RAPD-based molecular markers were scored visually using the software package MVSP (Multi-Variate Statistical Package) and DNA bands were scored as present (1) or absent (0). The pairwise comparisons between the tested isolates were used to calculate the coefficient of genetic similarity matrix (Gs) according to Nei and Li (1979). To convert the genetic similarity into genetic distance, logarithmic transformation (-Ln Gs) was computed to linearize the distance measure. Cluster analysis was presented as the dendrogram based on similarity estimates using the unweighted pair-group method with arithmetic average (UPGMA).

$$\text{Similarity} = \frac{2 * n11}{(2 * n11) + n01 + n10}$$

Where:

n11 - designates the number of common bands for the two compared samples,

n10 - cases where the bands were visible only in the first sample,

n01 - when bands were visible in the other sample only (Dice, 1945).

**Results and Discussion:**

- **Determination of antimicrobial activity:**

A total of 110 bacterial isolates were isolated from various soil samples obtained from different geographic regions. The isolates codes and their geographical regions are given in Table (2).

The data of the sensitivity of various tester strains **(**Gram-positive and Gram-negative**)** to the soil isolates by agar streak method are shown in (Table 3). Only ten isolates out of a total 110 bacterial isolates had broad spectrum antimicrobial activity against both Gram-positive and Gram-negative tester strains. These isolates were chosen for the further molecular differentiation.

**Table (2): Isolates codes and their geographical regions.**

| Code name | Geographical region of isolates |
|---|---|
| From **D1** to **D30** | Cairo |
| From **D31** to **D49** | Qena |
| From **D50** to **D62** | Assiut |
| From **D63** to **D84** | New Assiut City |
| From **D85** to **D110** | Qena |

**Table (3): Sensitivity of various tester strains to the soil isolates by Agar Streak Method.**

+++ = Very good, ++ = Good inhibition, + = Moderate inhibition, - = No inhibition.

| Soil bacterial isolates | Bacterial tester isolates | | | |
|---|---|---|---|---|
| | ***B. Subtilis*** | ***S. aureus*** | ***E. coli*** | ***P. aeruginosa*** |
| **D** 3, 55, 42 | ++ | ++ | + | _ |
| **D** 103 | + | ++ | _ | _ |
| **D** 106, 29 | ++ | +++ | ++ | +++ |
| **D** 110, 40, 65 | +++ | +++ | + | _ |
| **D** 104 | ++ | _ | ++ | _ |
| The rest of the isolated strains | Gives moderate inhibition with just one or another of the tested strains. | | | |

As shown in Table (3) it can concluded that the tester strains, namely; *B. subtilis* and *S. aureus* (Gram positive) were more sensitive for bacterial soil isolates than *E. coli* and *P. aeruginosa* (Gram negative). The data also indicates that the lowest sensitivity to bacterial soil isolates linked to the tester strain *P. aeruginosa*, whereas the high sensitivity of the test strains for soil isolates were noted for *B. subtilis* . Fitri and Bustam (2010) concluded that the Gram negative bacteria usually have better protection to other antimicrobial compound rather than gram positive bacteria because both kinds of bacteria have different cell wall components , cell wall of Gram positive bacteria contains peptidoglucan while cell wall of gram negative bacteria contains peptidoglucan and lipopolysaccharides.

Five strains that showed promising results for an effective antibiotic production through the microbial sensitivity tests were selected and identified as D110, D103, D104, D106, and D3. The characteristics of such strains are shown in Table (4). The five coded selected strains were identified as, *Bacillus licheniformis, Bacillus pumilus, Staphylococcus epidermidis, Serratia marcescens* and *Paenibacillus lautus* as shown in Table 6 (Doaa, *,et al.,* Personal communication).

**Table (4): The characteristics of the five selected isolates D110, D103, D104, D106, and D3.**

| Characteristics Strains | Form | Surface | Margin | Elevation | Colour | Cell shape | Gram stain |
|---|---|---|---|---|---|---|---|
| **D3 (*Bacillus pumilus)*** | Irregular | Smooth | Undulating | Flat | Yellowish | rod | + |
| **D 103 (*Staphylococcus epidermidis)*** | Circular | Rough | Entire | Convex | Whitish | coccus | + |
| **D104 (*Paenibacillus lautus*)** | Circular | Rough | Entire | Convex | Whitish | rod | - |
| **D 106 (*Serratia marcescens*)** | Circular | Smooth | Entire | Convex slightly unbonate | Red pigment | rod | - |
| **D 110 (*Bacillus licheniformis*)** | Irregular | Rough | lobate | unbonate | Whitish | rod | + |

• **Genetic variation between the isolates by using RAPD-PCR analysis:**

RAPD-PCR analysis technique has been used to differentiate among the five selected antibiotic producing isolates. Figure (2) gives the Agarose gel electrophoresis of RAPD products obtained by the different primers. RAPD-PCR produced banding patterns with products ranging from approximately 95 to 977bp. The total number of bands produced was 45, from which 19 bands were polymorphic with polymorphism percentage 42.22% (Table 5). The high polymorphism may be due to different sources/ locations of the of the isolates (Table 5).

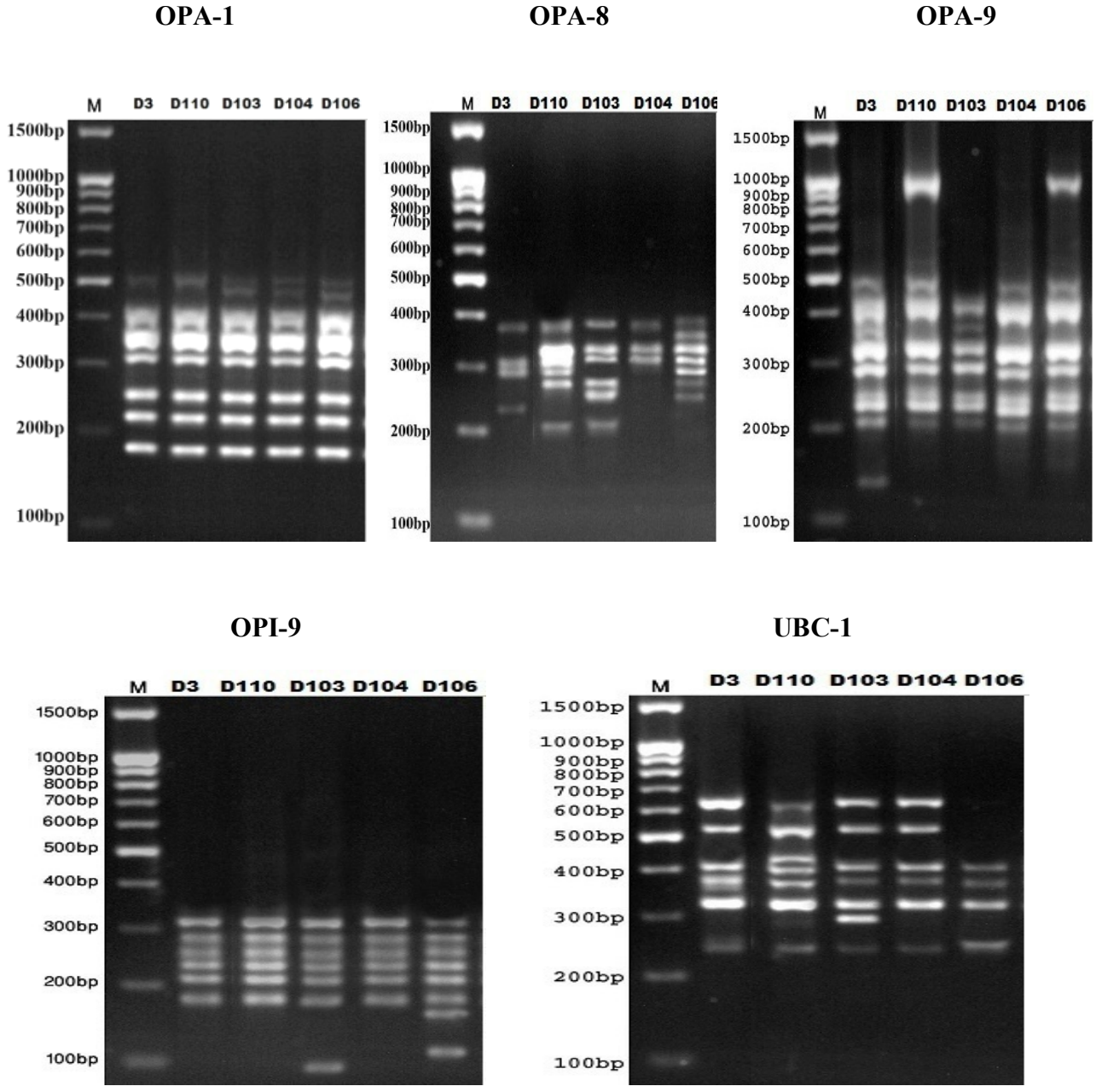



**Fig. (2):** Agarose gel electrophoresis of RAPD products obtained by OPA-1, OPA-8, OPA-9, OPI-9, and UBC-1 primers.

**Table (5): Polymorphism obtained by RAPD analysis among the selected isolates of antibiotic producing bacteria using five different primers.**

| primer | Total number of bands (a) | Number of polymorphic bands (b) | Polymorphism -b/a * 100% |
|---|---|---|---|
| **OPA-1** | 8 | 1 | 12.5 |
| **OPA-8** | 9 | 7 | 77. 78 |
| **OPA-9** | 10 | 4 | 40 |
| **OPI-9** | 9 | 3 | 33.33 |
| **UBC-1** | 9 | 4 | 44.44 |
| **Total** | 45 | 19 | 42.22 |

- **Genetic similarity matrix and cluster analysis**

Data of the presence /or absence of DNA fragments (markers) phenotypically analyzed using MVSP program of Nie and Li (1979), and pairwise comparisons between the tested isolates were used to calculate the genetic similarity. Then, based on the calculated genetic similarity values presented in Table (6), an estimation of the relationship between the different isolates was concluded. Data showed that the lowest genetic similarity was observed between isolate D3 and D106 (80.6%), while the highest value was found between D110 and D104 (90.6.1%).

**Table (6): Genetic similarity values calculated from 45 DNA fragments generated with five primers in five bacteria strains.**

| | D3 | D110 | D103 | D106 | D104 |
|---|---|---|---|---|---|
| **D3** | 1 | | | | |
| **D110** | 0.879 | 1 | | | |
| **D103** | 0.836 | 0.870 | 1 | | |
| **D106** | 0.806 | 0.870 | 0.829 | 1 | |
| **D104** | 0.903 | 0.906 | 0.892 | 0.862 | 1 |

The five promising isolates of bacteria were grouped in four clusters based on genetic similarity given in Table (6), and dendrogram as in Figure (3). The first and second groups were represented by D106 and D103 respectively. The third group included the two isolates D104 and 110. The fourth group was represented by D3 strain.

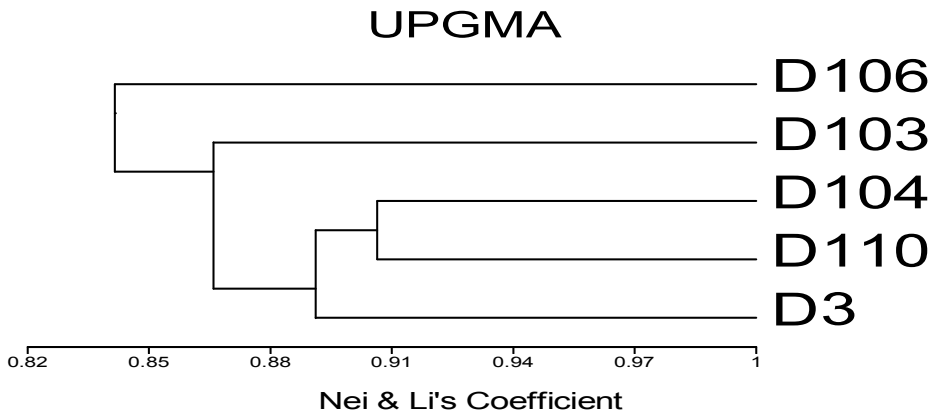



**Fig. (3):** Dendrogram of five bacteria strains developed from RAPD data using UPGMA analysis. The scale is based on Nei and Li coefficients of similarity.

The RAPD pattern technique permitted discrimination all reference isolates. The results show that RAPD method can reliably distinguished between selected isolates. Therefore, the method is well suited for discriminatory identification. RAPD technique has been described as a useful technique for both identification and typing of wine *lactobacillus* strains (Du Plessis and Dicks, 1995; Rodas, *et al*., 2005; Sohier, *et al*., 1999; Bartowsky, *et al*., 2003) and *Oenococcus oeni* (Zavaleta, *et al*., 1997). RAPD analysis makes comparable the whole chromosomal DNA exhibiting bands of genotypically specific value, and additionally this method turned out to be a fast, reliable, highly sensitive, and convenient method (Molnár, *et al*., 1993). Thanos, *et al*., (1996) proved that the numbers and sizes of amplification products were characteristic for each species .

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# تحليل العلاقة الوراثية للبكتيريا المنتجة للمضاد الحيوى المعزولة من التربة الملوثة بالفضلات الآدمية باستخدام تقنية الـ DNA ( RAPD )

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يعتبر المضاد الحيوى الذى تنتجه الكائنات الدقيقه المختلفه (مثل البكتريا والفطريات)– أحد أهم النواتج الآيضية الثانوية التى تستخدم على نطاق تجارى ، والتعرف المتنامى تحت ضغط الحاجه كمواد جديدة مضادة للميكروبات لمعالجة الأمراض المعدية يتطلب إكتشاف سلالات بكتيرية جديدة منتجة للمضادات الحيوية.

وقد أستهدف البحث الحالى عزل بعض السلالات من البكتيريا المنتجة للمضادات الحيوية من التربة الملوثة بالمخلفات الآدمية والتفرقة بين هذه السلالات بإستخدام تحليل RAPD-PCR.

في هذه الدراسة، تم جمع مائة وعشرة سلالات من العزلات البكتيرية من مناطق جغرافية مختلفة في مصر من التربة الملوثة بالمخلفات الآدمية . وأظهرت الخمسة عزلات المختارة (D110، D3، D103، D106 وD104) نتائج واعدة لإنتاج مضاد حيوي فعال من خلال اختبارات حساسية هذه العزلات مع سلالتين من البكتيريا الموجبة لصبغة جرام و سلالتين من البكتيريا السالبة لصبغة جرام من خلال تكوين منطقة رائقة نتيجة تثبيط النمو بها . وقد أظهرت النتائج أن متوسط إتساع هذه المناطق فى حالة إستخدام البكتيريا الموجبة لجرام أكبر مما هو فى حالة إستخدام البكتيريا السالبة لجرام مما يدل على أن العزلات أكثر قدرة على تثبيط نمو البكتيريا الموجبة جرام بالمقارنة مع تثبيطها لنمو البكتيريا السالبة جرام.

بالإضافة إلى ماسبق ، فقد أمكن أن نفرق بين هذه السلالات الخمسة المعزولة – على المستوى الجزيئي باستخدام تحليل RAPD-PCR ، وقد أظهرت النتائج أن هذه العزلات يمكن وصفها طبقا لأنماط أشكالهم الحزمية ، وقد أعيد تجميعها فى أربع مجموعات على أساس تشابهاتها الوراثية المعينة . وقد مثلت المجموعات الأولى والثانية العزلات D106 و D103 على الترتيب ، بينما ضمت المجموعة الثالثة اثنين من العزلات هما D104 و D110 ، أما المجموعة الرابعة فقد تمثلت فى السلالة D3.