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Use of Repetitive DNA Sequences and the PCR to Identify Fecal *Escherichia coli* Isolates from Different Soils

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Abstract: ***Microbial communities which inhabit the soil environment are large and diverse. Because of the tight relationship between microbial activity and soil functioning and the reality that microorganisms are sensitive and respond quickly to changes in soil conditions. Fecal coliforms normally inhibited the intestinal tract of warm-blooded animals. Outside of a warm-blooded host, fecal coliforms are short-lived in comparison with the coliform bacteria that are free-living and not associated with the digestive tract of man or animals. Gram negative microorganisms have been reported to be capable of utilizing a wide variety of hydrocarbons as carbon and energy source such as E.coli. The aims of present study are the Isolation and identification of Escherichia coli isolates from (Hydrocarbons & Sewage) contaminated soils.In this paper six soil samples (Contaminated with Hydrocarbons, Sewages and control) were collected from different regions in Baghdad City. And a total of 57 bacterial isolates were isolated from these soils that belong to Escherichia species by making Gram staining, cultured on MacConkey and EMB agar to observe the colony morphology, biochemical tests were used in the identification of bacteriaincluded: (Thermotolerant (faecal) Coliform test, Indole test, Methyl red test, Voges – Proskauer test, Citrate utilization test, Motility test), And because of E. coli is highly diverse at the genotypic level as well. This High genotypic diversity has been identified in E. coli based on the repetitive extragenic palindromic PCR (rep-PCR) DNA fingerprinting (BOX-PCR). This technique can reveal the alteration of microbial genome structures and evolution of microbial genomes. The result shows a lot of genetic variation among isolates from the same and different soils. This lead to the possibility of using the E.coli as indicator organism and because of the tight relationship between the status of microorganism and soil functioning, and also the fact that microorganisms respond quicker to changes in soil environment, E.coli is important to early detect and assess changes in soil status, in order to determine the direction of that change.***

Keywords: *E. coli,* rep-PCR, BOX-PCR, IMViC.

1. Introduction

Soil is a complex and dynamic habitat [1].Its physical, chemical and biological characteristics are determined by the relative proportion of those components, markedly influenced by environmental factors and biotic activities over time [2]-[3].While Soil characteristics, in turn determine the natural vegetation, as well as mesofauna and microbial community structure and activity [4]. The survival and inactivation of pathogenic microorganisms in the soil depend on environmental factors, such as precipitation and solar radiation; physicochemical factors, such as soil texture and structure, water flow in the soil (saturated and unsaturated), humidity, temperature, organic matter content and pH; and biological factors, such as the type of microorganism, antagonistic interactions such as predation, competition and the production of inhibitory substances that may reduce their concentration in the soil [5].

Oil contamination has badly affected the lives of many people living in areas near oil exploration sites. With crude oil and production chemicals polluting water supplies, soil, air, and surrounding plant and animal life, human health has suffered.

A number of gram positive and negative microbes have been reported to be capable of utilizing a wide variety of hydrocarbons as carbon and energy [6]. The microorganisms include bacteria of the genera *Klebsiella*, *Proteus*, *Bacillus*, *Escherichia*,*Pseudomonas*, *Streptomyces*, *Nocardia*, *Seratia*, *Xanthomonas*, *Micrococcus* etc. and fungi of the genera-*Rhizopus*, *Fusarium*, *Pencillium*, *Cladosporium*and Aspergillus etc. [7]. The hydrocarbon degrading microbes have an inherent capacity to assimilate hydrocarbons and/or its products [8]. The rate and extent of biodegradation can be affected by some main factors which are: characteristics of the oil, the availability of oxygen and nutrients (nitrogen and phosphorus) and temperature.

In order to have a more comprehensive study,we had collect soil samples that contaminated with sewage, and because of that Sewage sludge was found to be an effective organic fertilizer for many crops [9] and the recycling of sludge for agricultural purposes is an alternative to handle its increasing quantities produced in recent years [10]. Soil amendment with sewage sludge has become an agricultural practice of heightened interest, as a consequence of the increase in sewage sludge production [11].

Members of two bacteria groups, coliforms and fecal streptococci, are used as indicators of possible sewage (fecal) contamination because they are commonly found in human and animal feces. Moreover, they are generally not harmful themselves; they indicate the possible presence of pathogenic (disease-causing) bacteria, viruses, and protozoans that also live in human and animal digestive systems. Though, their presence in streams suggests that pathogenic microorganismsmight also be present and that swimming and

eating shellfish might be a health risk. Since it is difficult, time-consuming, and expensive to test directly for the presence of a large variety of pathogens, water is usually tested for coliforms and fecal streptococci instead. Escherichia coli are a commoninhabitant of the gastrointestinal tract of humans and animals. There are E. coli strains that are harmless commensals of the intestinal tract and others that are major pathogens of humans and animals.

The pathogenic *E.coli* is divided into those strains causing disease inside the intestinal tract andothers capable of infection at extra 342 intestinal sites [12].

*Escherichia coli*extraintestinal infections (urinary tract infections and bloodstream infections) represent a significant public health burden worldwide [13]-[14]. Since the 2000s, antimicrobial resistance among *E. coli* isolates has increased contributing to the complexity in management of such infections [15]-[16].

This is a particular version of repetitive extragenic palindromic-PCR (rep-PCR) that uses the BOX-A1R primer. BOX-PCR is a fingerprinting analysis based on the BOX dispersed-repeat motif, firstly identified in *Streptococcus pneumoniae*, but common in a number of bacterial species. Since the BOX repetitive sequences are interspersed throughout the genome, BOX-PCR is a method potentially capable of simultaneously surveying many DNA regions scattered in the bacterial genome. It has been shown to have similar or even better strain differentiation power, as well as to be easier to perform, than other fingerprinting methods. BOX-PCR is quicker, cheaper, and in many cases more discriminatory than pulsed field gel electrophoresis, despite is generally less reproducible. BOX-PCR patterns are not affected by the culture age of the strain to be analyzed and fingerprinting output can be easily analyzed by computer assisted methods [17].

E. coli is highly diverse at the genotypic level as well. High genotypic diversity has been identified in E. coli based on the repetitive extragenic palindromic PCR (rep-PCR) DNA fingerprinting [18]-[19]-[20]-[21]. Such genotypic diversity appears to be common among environmental strains [22]-[23]. Whole genome typing methods such as the rep-PCR DNA fingerprintingTechnique can reveal the alteration of microbial genome structures, which allows these techniquesto be used in studying plasticity, molecular phylogeny, and evolution of microbial genomes [24].

2. Materials and Method

2.1 Samples Collection

In this papersix soil samples (Contaminated with Hydrocarbons, Sewages and control) were collected from different regions in Baghdad City. And a total of 57 bacterial isolates were isolated from these soils that belong to *Escherichia* species through the period from October 2016 to December 2016, Table (2-1).

Table (2-1): Sampling location and number of samples and isolates of each location site

Locations	Number of samples	Number of isolate
Al-Khadumya	1	8
Hay Al-Adel	1	8
Al-Hurria	1	12
Al-Otaifia	1	12
Hay Al-jamiaa	1	8
Hay Al-salam	1	9
Total	6	57

2.2 Identification of Bacteria

For all soil samples, suspension was prepared by adding 10g (dry weight) of soil sample to 90ml of sterile distilled water, This will be dilution 1:10, mix well and stand for 5 minute in order to precipitate soil particles. Later, 1ml was transferred (using a sterile pipette) to another tube contain 9ml sterile distilled water to make dilution number 2, and so on to make 10 serial dilutions. Then 1ml from each dilution was transferred in the center of sterile petri dishes using a sterile pipette. MacConkey agar is then poured into the petri dishes containing the inoculum and mix well clockwise and anti-clockwise. After the solidification of the agar, the plates is inverted and incubated at 37°C for 24-48 hours. Later sup-culturing was made several times to get pure culture [25].

And later, the isolates subcultured on EMB agar to observe the colony morphology (shape, size, surface texture, edge and elevation, color, opacity etc). The organisms showing characteristic colony morphology of *E. coli* was repeatedly subcultured onto EMB agar until the pure culture with homogenous colonies were obtained. And also based on taxonomic tables, and for further conventional diagnosis, some of the biochemical tests were used in the identification of bacteria [26]-[27] included: Thermotolerant (faecal) Coliform test, Indole test, Methyl red test, Voges –Proskauer test, Citrate utilization test, Motility test.

a) Thermotolerant (faecal) Coliform test
The term “fecal coliform” has been used in microbiology to denote coliform organisms which grow at 44 or 44.5 C and ferment lactose to produce acid and gas. Prepare MacConkey purple broth media in test tubes with Durham’s tube and autoclave it. Inoculate the tubes with loopfull of bacteria; incubate the tubes at 37˚C and 44˚C for 24 hours. After incubation observe the gas production in Durham's tube and color change of media [28].

b) Indole test
Peptone water broth prepared as mentioned by [29]was inoculated with overnight tested bacterial culture and incubated at 37°C for 24 hrs, after that 10 drops of Kovac 's reagent was added directly to the culture tube, the immediate formation of a red ring at the top of the broth after gentle shaking indicated positive result and if not this indicated the negative result.

c) Methyl red test
MR-VP medium was prepared according to the manufacturer company and inoculated with the tested bacterial culture and incubated at 37 °C for 24 hrs, a few drops of methyl red solution were added to the broth culture, if the color of

medium change from yellow to red this indicated a positive result, while if the medium stay yellow this indicated a negative result.

d) Voges –Proskauer test

MR-VP medium prepared according to the manufacturer company and was inoculated with the tested bacterial culture, incubated at 37°C for 24 hrs, then 0.6ml of VP1 (α-naphthol solution) and 0.2ml of VP2 (Potassium Hydroxide solution) previously prepared were added; red color after 15 min. indicated a positive result while the negative result confirmed when the color of the reagent remain unchanged [30].

e)Citrate utilization test

Simmon's citrate agar slant prepared as mentioned in the manufacturer company was inoculated with tested bacterial culture by streaking with sterile loop and incubated at 37°C for 24-48 hrs, positive result was indicated by changing the color of the medium from green to blue while green color of the medium remains the same if it is negative result. [31].

f) Motility test medium

This medium composed of: (Agar-agar 0.4g and Nutrient broth 0.8g) Dissolved in 100ml D.W., boiled for 1 min., sterilized in autoclave at 121°C /15 pounds/inche² for 15 minutes and dispensed in small sterile vials. It was used for the detection of bacterial motility by stabbing method [32].

2.3 DNA Extraction

Heat lysis method: 0.5 ml of overnight *E.coli* culture was centrifuged and the supernatant was removed. The cell pellet was washed once with 0.5 ml of sterile water, resuspended in 100 µl of sterile water, and heated at 95˚C for 10 minutes [33]. A slight variation of the method was made as well, as described below. 1.5 ml of overnight *E.coli* broth culture was centrifuged. Following removal of the supernatant, the cell pellet was washed once with 0.5 ml of sterile water, resuspended in 50 µl of sterile water, and then heated at 95˚C for 10 minutes. DNA Samples were stored at – 20 C˚ until use. 1µl of cell suspension was used per PCR reaction. Later NanoDrop Spectrophotometer was used to measure the DNA concentration and purity, DNA concentration was about 300-400 ng/µl while, the purity between1.9-2.0 is usually accepted.

2.4 Primer Selection and Preparation

Oligonucleotide primer was prepared depending on the manufacturer's instructions .The primer (Alpha DNA, Canada) was provided in lyophilized form, dissolved in sterile deionized distilled water to give a final concentration of 100 picomole/µl as recommended by the provider and stored in a deep freezer until use. The primer used in this study was shown in table (2-2).

Table (2-2): Oligonucleotide primer sequences used for PCR amplification of rep. PCR

Genes	Sequence (5' to 3')	Size (bp)
BOX A1R Primer	(5'-CTACGGC AAGGCGACGCTGACG -3')	

2.5 Amplification Reaction

The extracted DNA, primers and Master Mix (Bioneer (Korea)) contain: Taq DNA polymerase, MgCl2, deoxynucleotides dNTPs, KCl, stabilizer and tracking dye and Tris-HCl (pH 9.0) was thawed at 4°C, vortexed and centrifuged briefly to bring the contents to the bottom of thetubes. Optimization of polymerase chain reaction was accomplished after several trials, PCR mixture was set up in a total volume of 20 µl. 1 µl of BOX primer (100 picomole/ µl) and 2 µl of template DNA have been used. The rest volume was completed with sterile Deionized D.W. this PCR reactants are listed in Table (2-3). Negative control contained all material except DNA; D.W. was added instead of template DNA. PCR reaction tubes were vortexed and finally placed into thermocycler PCR instrument. DNA was amplified as indicating in Table (2-4).

Table (2-3): PCR Reactants Used

No.	Components	Volume (µl)
1	Template genomic DNA	2
2	Primer 100 pmol/µl	1
3	Blue Master mix	0.2
4	Deionized D.W	17
The final volume of all components		20

Table (2-4): PCR amplification program for BOX Primer [34].

Stage		Steps	Temperature (C˚)	Time (Min.)	No. of Cycles
First		Initial Denaturation	94 C˚	2 min.	1
Second	I	Denaturation	94 C˚	1 min.	35
	II	Annealing	55 C˚	1 min.	
	III	Extension	72 C˚	1 min.	
Third		Final extension	72 C˚	7 min.	

2.6 Agarose Gel Electrophoresis

A concentration of 0.01% agarose was used in electrophoresis after DNA purification and after PCR detection. 0.01% Agarose gel was prepared by dissolving 0.55 gm of agarose powder in 45 ml of (1X) TBE buffer (pH 8) on hot plate with magnetic stirrer, left until boiling and becoming clear, allowed to cool to 50˚C and 2 µl RedSafeTM (Nucleic Acid Staining Solution) was added.

2.7 Analysis of PCR results

PCR product was analyzed by gel electrophoresis in 0.01% agarose containing RedSafeTM (Nucleic Acid Staining Solution). 2 µl from amplified sample was directly loaded in agarose gel. 6µl DNA Ladder (1000bp) as standard in electrophoresis and the gel was run at 80 Volt for 55 minutes. The products were visualized with UV illuminator and imaged.

3. Results

3.1 Conventional Methods

All the samples were primarily diluted in sterilized Distilled water (serial dilution was made), then cultured onto the MacConkey agar by Pouring Plate method [35]. And later,

all isolates subcultured on EMB agar to observe the colony morphology (shape, size, surface texture, edge and elevation, color, opacity etc). The organisms showing characteristic colony morphology of *E. coli* was repeatedly subcultured onto EMB agar until the pure culture with homogenous colonies were obtained. The colonies of *E.coli* on MacConkey agar appeared as small pink colonies (Lactose fermenter) as shown in figure (3-1), whereas on EMB agar appeared as green metallic sheen figure (3-2).

Lactose- and indole-positive colonies were presumptively identified as *E.coli*[36]. EMB agar provides favorable conditions for growth of *E. coli* and improves its proliferation in particular compared to other lactose-positive bacteria.

Escherischia coli produced huge amounts of acids from lactose that resulted in very darkmetallic shiny colonies in which *Klebsiella*and *Enterobacter* were lacking. This might be due to less acid accumulation that caused the appearance of pink colonies. Procedures described by [37]. The organisms revealed gram negative, pink colored with rod shaped appearance and arranged in single or in pair were suspected as *E. coli.*

Figure (3.1): Pink colony of *E.coli* on MacConkey agar

Figure (3.2): Green metallic sheen of *E.coli* on EMB agar

All green metallic sheen and lactose fermenting isolates were tested for several biochemical tests that showed in table (3-1). The results of biochemical tests were compared with characteristics of *E.coli*documented by standard methods [38]-[39], *E. coli*, in particular, was identified as follows: Gram-negative bacilli (presumptively Family Enterobacteriaceae) catalase positive, oxidase-negative, Indole positive, MR positive, VP negative, citrate negative. Isolates that were citrate-positive, VP-positive can be representing other spp.of Enterobacteriaceaea.

Table (3.1): Diagnosis tests results for *Escherichia coli*

Id	Biochemical test	Results
1	Gram stain	-
2	Green metallic sheen producing	+
3	Citrate utilization	-
4	Lactose fermentation	+
5	Indole production	+
6	VogasProskauer test	-
7	Methyl red	+
8	Motility	+

3.2 Loading and running DNA in agarose gel

Five µl of each DNA template was transferred to Eppendorf tube, 3µl of loading dye was added to the tube and the mixture was loaded into the wells in agarose gel. The electric current was allowed at 80 volt for 50 minutes. UV transilluminater was used for the observation of DNA bands, and the gel was photographed.

3.3 Molecular Genotyping

BOX-PCR typing was carried out to differentiate precisely among the 57 E. coli isolates taken from six locations in Baghdad city with two control Soil groups (C & NC). (C sample was taken from The Tigris River Bank in Al-Otaifia city) included 12 isolates, while (NC sample was taken from roadside garden in Hay Al-jamiaa city) included 8 isolates. The first sample (H) (Taken from nearby electric generator in Al-Khadumya city) included 8 isolates. The second sample (NH) (Taken from nearby electric generator in Hay Al-salam city) included 9 isolates. The third sample (S) (Taken from area nearby septic tanks in Al-Hurria city) included 12 isolates. And the last sample (NS) (Taken from area nearby septic tanks in Hay Al-Adel city) included 8 isolates. PCR-based typing methods have a widespread and important role in studies of environmental, agricultural, medical and industrial microbial ecology. The rep-PCR genomic fingerprints generated from bacterial isolates permit differentiation to the species, subspecies, and strain level.The band profiles of the amplified repetitive regions are unique among species, or even between species, thus different species can be identified through their band patterns.

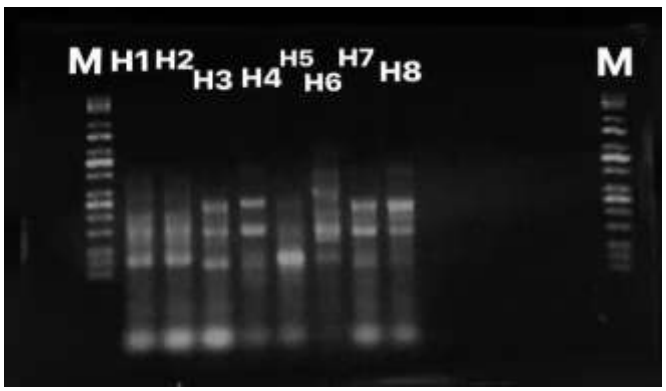

Figure (3.3.A): BOX-PCR fingerprinting of *E.coli* isolates (Hsampletaken from nearby electric generator in Al-Khadumya city included 8 isolates).

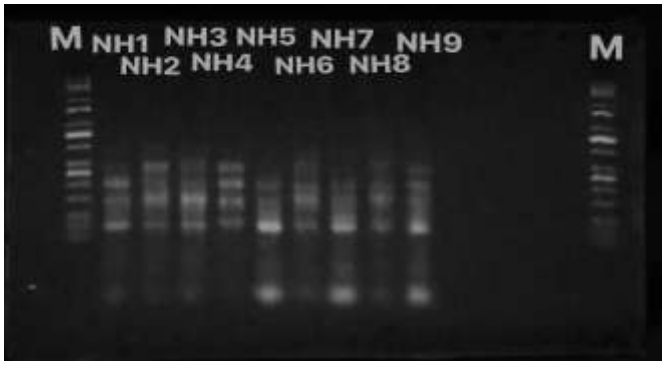

Figure (3.3.B): Box-PCR fingerprinting of *E.coli* isolates (NH sample taken from nearby electric generator in Hay Al-salam city included 9 isolates).

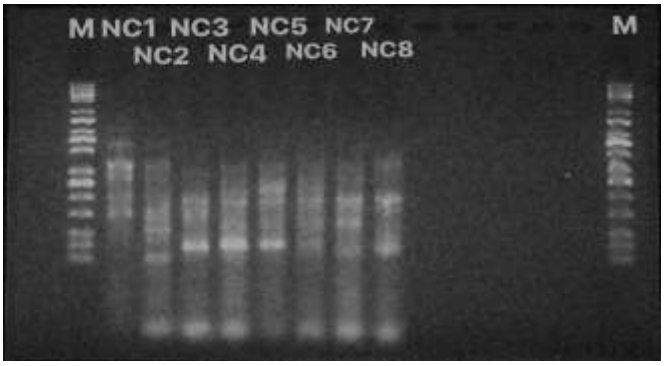

Figure (3.3.F): Box-PCR fingerprinting of *E.coli* isolates (NC samplewas taken from roadside garden in Hay Al-jamiaa city included 8 isolates).

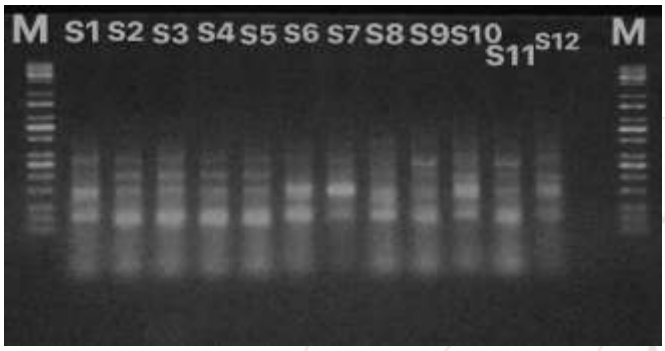

Figure (3.3.C): Box-PCR fingerprinting of *E.coli* isolates (S sample taken from area nearby septic tanks in Al-Hurria city included 12 isolates).

Figure (3.3: A, B, C, D, E, F): BOX-PCR fingerprint patterns of 57 environmental *E.coli* isolates. Bands of gel images were detected by gel electrophoresis on agarose gel (0.01%) containing RedSafeTM (Nucleic Acid Staining Solution) and the gel was run at 80 volt for 55 minute, and visualized on a UV transiluminator documentation system. M represents DNA ladder (1000 pb), Lanes (H1-NC8) signify the all 57 *E.coli* isolates.

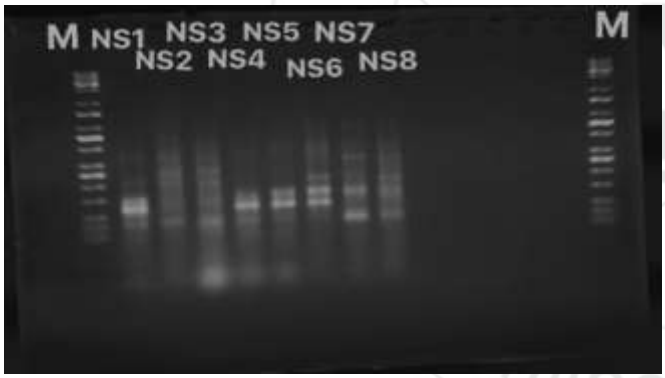

Figure (3.3.D): Box-PCR fingerprinting of *E.coli* isolates (NS sample taken from area nearby septic tanks in Hay Al-Adel city included 8 isolates).

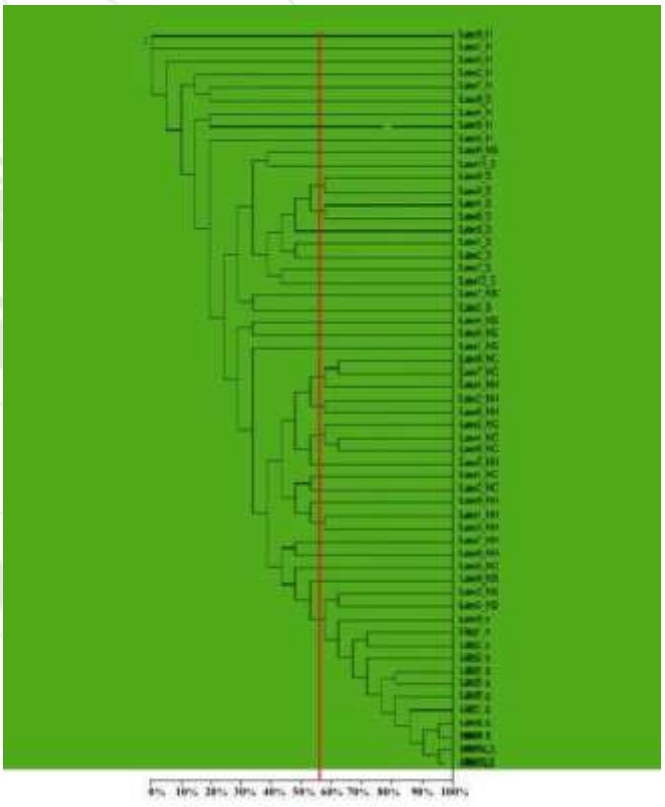

Figure (3.4): Dendrogram (Cluster analysis) using Box-PCR fingerprint patterns of 57 environmental *E.coli* isolates. Clustering on a 56% similarity

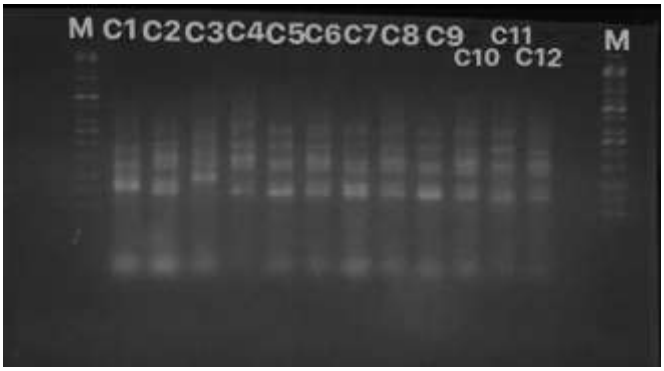

Figure (3.3.E): Box-PCR fingerprinting of *E.coli* isolates (C sample was taken from The Tigris River Bank in Al-Otaifia city included 12 isolates).

Only seven clusters have been found based on 56% cut off similarity: Cluster I covers isolates (0-S and 5-S) ; while cluster II includes isolates (4-S and 6-S). Isolates (0Nc, 7Nc and 4-NH) were involved in cluster III ., cluster IV comprised isolates (2NH and 6NH)., cluster V comprised isolates (3,4,6 Nc).Cluster VI covers isolates (1,3 NH).Cluster VII includes isolates (2,3 Ns and 0,1,2,3,4,5,6,7,8,9,10,11C).

4. Discussion

The result of the conventional methods (Biochemical tests) showed diagnosis of the isolates according to the taxonomic tables. *E.coli* showed Indole positive result due to the bacterial capacity to produce treptophanase enzyme which hydrolyze treptophane to indole, pyrovic acid and ammonia. Indole production is detected by Kovac's or Ehrlich's reagent which contains 4 (p)-dimethylamino benzaldehyde, this reacts with indole to produce a red coloured compound. Also Methyl Red (MR) test determines whether the microbe performs mixed acids fermentation when supplied glucose. So, *E.coli* showed positive MR When the culture medium turns red after addition of methyl red, because of a pH at or below 4.4 from the fermentation of glucose.The Voges-Proskauer (VP) test is used to determine if an organism produces acetylmethylcarbinol from glucose fermentation. If present, acetylmethylcarbinol is converted to diacetyl in the presence of ∝- naphthol, strong alkali (40% KOH), and atmospheric oxygen. The ∝-naphthol was not part of the original procedure but was found to act as a color intensifier and must be added first. The diacetyl and quanidine-containing compounds found in the peptones of the broth then condense to form a pinkish red polymer. While, *E.coli* gives negative result to Vogas Proskauer testbecause that *E.coli* is unable to produces acetylmethylcarbinol. Moreover, Citrate utlilization test is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic (NH4H2PO4) is the sole fixed nitrogen source.*E.coli* gives negative Simmon Citrate test, no color change will occur; the medium will remain the deep green color of the uninoculated agar. Only bacteria that can utilize citrate as the sole carbon and energy source will be able to grow on the Simmons citrate medium, thus a citrate-negative test culture will be virtually indistinguishable from an uninoculated slant. And by stabbing method *E.coli* were tested for motility. *E.coli* is motile with peritrichous flagella. The result of the Repetitive sequence-based PCR (rep-PCR) genomic fingerprinting shows the genetic relatedness between *E.coli* isolates. Oligonucleotide primers have been designed to prime PCR-mediated DNA synthesis outward from the box A subunit of BOX [40]. The use of these primer(s) and PCR leads to the selective amplification of distinct genomic regions located between BOX elements. The amplified fragments can be resolved in a gel matrix, yielding profiles referred to collectively as rep-PCR genomic fingerprints. The rep-PCR genomic fingerprints generated from bacterial isolates permit differentiation to the species, subspecies, and strain level. And because of the BOX-PCR genomic fingerprint profiles were sufficient to determine the sources of fecal pollution [41], it had been used in this study. The result in this studyshows 56% cut off similarity.This mean there is a lot of variation between the 57 isolates from the different 6 soils. The benefits of molecular methods are more sensitive, more qualitative for results, materials available, but the drawback of molecular methods is costly. These explanations made molecular methods relatively more accurate than conventional methods [42]. The data of the present study agrees with the study conducted by[43] that related to the Subtyping of foodborne and environmental isolates of *Escherichia coli* by multiplex-PCR and rep-PCR.

5. Conclusion

This study has demonstrated the efficiency of the rep-PCR (BOX-PCR) fingerprinting as molecular method to detect the genetic relatedness between fecal *E.coli* isolates from different soils and different environmental pollutants.In addition, the present study referred to the possibility of using the BOX-PCR fingerprinting test as rapid, specific and sensitive method to prevalence the fecal *E.coli* in the environment.

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