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Original Research

Bacteriocin Production by *Escherichia coli* Isolated from Clinical Samples

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Abstract:

The issue of antibiotic resistance has been becoming worse recently, therefore it's critical to discover new treatments for diseases brought on by microorganisms that current medications are unable to eliminate. Bacteriocin, a class of small antimicrobial peptides (AMPs), has demonstrated efficacy against a wide range of multidrug-resistant microorganisms, suggesting that it may be a viable antibiotic substitute. Apart from its minimal cytotoxicity against human cells and strong selectivity against microbial infections, the majority of bacteriocins exhibit stability and resilience to pH and temperature changes as well as enzyme breakdown. Small peptides known as bacteriocins have a wide range of structures and purposes, but little is known about how they work. The bacteriocin that is created by *E. coli* will be discussed in this study, along with its modes of action, the culture conditions that are employed to enhance its production, and the cutting-edge methods that are utilized to discover them. The use of bacteriocin as novel cancer treatment agents and food preservatives will also be covered.

Keywords: Escherichia coli, Clinical Samples, Bacteriocin Production

Introduction:

The bacteriocins are protein compounds with a large molecular weight, which have antibacterial activity and are produced by bacteria and inhibit or kill other bacterial species. They are produced by different bacterial groups. These antibiotics differ from each other in size and methods of secretion from the producing cells, as well as in the methods of their transfer into sensitive cells (Ridley and Lakey 2015). The production of bacteriocins is generally under plasmid control (Harry and Walker 2013), however, there are bacteriocins whose production is under chromosome control, especially Microcins, such as Bacterocin 28b produced by Serratia marcescens, whose genes are carried on the chromosome, and this was confirmed by the researcher Cursino and his group (Cursino, Šmarda *et al*. 2002).

Bacterocins are produced and stimulated in bacteria that have a special plasmid to produce bacteriocins only, and their production is normally in small quantities (Pugsley and Schwartz 1983), but the production of these proteins can be increased Dozens or hundreds of times by using stimulants such as mitomycin-C (Mt-C) (Cascales, Buchanan *et al*. 2007), or some antibiotics such as Chlormephincol (Pugsley and Schwartz 1984), globomycin (Cavard, Baty *et al*. 1987), or Ciprofloxacin (Jerman, Butala *et al*. 2005) are used for the same purpose. Hydrogen peroxide (H2O2) and some types of dyes may also be used (Bradley 1967). Ultraviolet rays were used in other studies to stimulate production, as well as heat (Cascales, Buchanan *et al*. 2007). As for the researcher Bures and his group, they used thymine to stimulate production in glass (Bures, Horák *et al*. 1986). On the other hand, the production of colicin can be stimulated automatically, as confirmed by the researcher Pugsley (Pugsley 1983). Despite the multiplicity of stimulating materials, whether physical or chemical, the principle of its operation is the same, as the manufacturing process is under the control of the (SOS system) and may be called the SOS Repair System or the SOS Operon System, which is a complex enzyme system (Mader, von Bronk *et al*. 2015).

Escherichia coli is a member of the Enterobacteriaceae family, Gram-negative, motile or non-motile rod-shaped, lactose fermenter, the optimum temperature for its growth is (36-37)ºC (Wanger, Chavez *et al*. 2017). It is positive for catalase and methyl red tests and negative for oxidase and Vogase-Proskauer, does not consume citrate and produces indole (Hemraj, Diksha *et al*. 2013).

The pathogenicity of this bacterium is due to its possession of many virulence factors, including iron chelators, toxic necrosis factor, and colicin, and its possession of surface structures such as flagella, capsule, and lipopolysaccharides (Terlizzi, Gribaudo *et al*. 2017). *E. coli* bacteria are characterized by their possession of multiple resistance to antibiotics (Laird 2016), as they are characterized by their high resistance to antibiotics due to their possession of resistance enzymes such as β-lactamase enzymes β-lactamases that confer resistance to beta-lactam antibiotics. These bacteria also have other mechanisms that give them resistance to antibiotics, such as changing the permeability of the cell membrane, changing the target site, and inhibiting protein synthesis (Kapoor, Saigal *et al*. 2017).

Methods

Samples collection

Forty specimens from different sources (urinary tract infection, stool, diabetic foot, wounds) were collected from peoples in the stages of all ages and both sexes, in Al-Hillah Surgical Teaching Hospital in Babylon province during the period from August 2023 to September 2023, the patients have clinical symptoms: swelling, redness, skin discoloration, skin sores, numbness, pain, tingling, foul smell, wounds and discharge of fluid or pus. The samples were transferred to the laboratory to culture and diagnose the bacteria causing the diseases.

Laboratory Prepare and Sterilization of culture media

All ready culture media agars, broths and indicators were prepared according to the manufacturing company instructions; the constituents were dissolved in distilled water (D.W), to dissolve all constituents completely and sterilized by autoclaving at item 121 ºC for 15 min. After cooled to 45-50 °C, each medium was dispensed into sterile petri dishes or test tubes as required, media stored in a refrigerator 4-8 ˚C until use to ensure their safety (Orekan, Barbé *et al*. 2021).

- **MacConkey Agar**

This media was prepared by weighing a 51.6 g of MacConkey and dissolving it in a 1000 ml D.W, then transferred it to a plastic petri plate after sterilizing it in an autoclave, this medium selective and differential for gram negative bacteria contains and bile salts, which inhibits gram positive bacteria growth (Klangsin 2000).

❖ **Eosine methyline blue (EMB)**

The 35.96 g of powder was melting in (1000 ml) of D.W, mixed well and heated to boil to dissolved, cooled at 45-50°C, then it is poured into petri dishes, this media selective used for *E.coli* (MacFaddin 2000).

❖ **Brain Heart Infusion Broth**

This media was prepared by weighing a 37.0 g of powder and dissolved it in a 1L D.W, heated to boiling in an autoclave and cooled at 45-50 °C, then poured into petri dishes, stored in refrigerator 4 ºC (MacFaddin 2000).

❖ **Muller-Hinton agar**

This media was prepared by dissolved 38.0 g in 1L distilled water, the mixture was boiled until the medium dissolved completely, cooled and poured into sterile petri plates (MacFaddin 2000).

❖ **Nutrient agar**

This medium was prepared like MacConkey agar media but the amount of this medium is (28.0 g /1L).

3.2.3 Reagents and dyes Solutions

❖ **Catalase Reagent**

Catalase agent which is a ready commercial 3% hydrogen peroxide (H_2O_2). This is used to ability of bacterial isolate to yield enzyme which causative degradation H_2O_2 releasing free O_2 (MacFaddin 2000).

❖ **Oxidase Reagent**

This reagent was utilized for gram negative bacteria and to detect the bacteria ability to produce oxidase enzyme (Kohlerschmidt, Mingle *et al*. 2021).

❖ **Kovacs Reagent**

The reagent was utilized for identify of tryptophanase enzyme, able to hydrolyze tryptophan, or unable to hydrolyze tryptophan (MacFaddin 2000).

Isolation of Pathogenic Bacteria

After incubation time, a single, pure, and clear colonies were isolated and sub-cultured on specific selective media (MacFaddin 2000).

Identification of the Bacterial Isolates

Bacterial isolates, firstly growth on the cultured media. Secondly the bacterial isolates identified by using the gram stain. Thirdly, using by biochemical tests. All the bacterial isolates were confirmed using Vitek-2 compact system (MacFaddin 2000). They were identified as follows:

✓ **Initial bacteriological diagnosis**

The phenotypic characteristics of the bacterial colonies growing 24-48 hours at 37°C, on the culture media were studied, the culture characteristics were diagnosed in terms of (colony shape, size, color, texture, smell and fermentation of lactose on MacConkey agar), In addition to microscopic examination, after staining them with Gram stain, then examining them with an optical microscope using an oil lens with a magnification of 100 x, observing the size and shape of the cells, the way they group together, and their interaction with Gram stain (Hijazi, Siepmann *et al*. 2024).

✓ **Microscopic Examinations**

Smear of bacterial cells on a glass slide with one drop of normal saline, was fixed by heat, the crystal violet dye was put on the fixed bacterial cells slide for (1-3) min before being rinsed manor with distilled water, the smear was treated with a drop of iodine solution for (1 min) before being rinsed with distilled water and decolorized with 95% ethanol for (30 sec), after that, slide was counter stained with safranin for (1-3 min) before being rinsed with distilled water to remove the excess color, the slide was examined under a light microscope (100x), to identify the shape and arrangement of bacterial cells and classification based on it reaction with stain as gram positive bacteria or gram negative bacteria (Miller 2024).

✓ **Biochemical Tests**

Biochemical tests for the diagnosis of bacterial isolates were performed as follows:

- **Catalase Test**

From each selective culture media single colony was smeared on the slide. One drop of 3% H_2O_2 was putted on the smear, if the reaction is positive a bubbles seen ,if the reaction is negative a bubbles no seen (Gomez, Jimenez *et al.* 2023).

- **Oxidase Test**

From each selective culture media single colony was smeared on the filter paper. Two or three drop of the tetra-methyl-phenylene di-amine di-hydrochloride reagent was put on the smear, if the reaction is positive a shifted to purple color is seen ,if the reaction is negative a color no seen (Tarrand and Gröschel 1982).

- **Indole Test**

Two to four drops of Kovács reagent were added to the peptone water medium and inoculated with bacterial colony at 37 °C for 24 h, if the reaction is positive a brilliant pink ring is seen, if the reaction is negative a ring no seen (Alqaisi 2023).

- **Citrate Utilization Test**

From each selective culture media single colony was added to simmons citrate agar and incubated at 37 °C for 24 h, if the reaction is positive a change in the color of the pH indicator from green to blue, and a negative result indicates green (MacWilliams 2009).

- **Urease Production Test**

From each selective culture media single colony inoculated on the agar slant and incubated 24 h, if the reaction is positive a change in the color from yellow to purple (Brink 2010).

- **Triple Sugar Iron (TSI) Test**

The single colonies were inoculated into agar slant containing a pH-sensitive dye (phenol red) and multiple sugars and incubated at 37°C for 24 h. If the reaction of alkaline/acid (red slant/yellow butt): It is refer to dextrose fermentation only. If the reaction an acid/acid (yellow slant/yellow butt): It indicates to dextrose, lactose and/or sucrose fermentation. An alkaline/alkaline (red slant, red butt) reaction: Absence of carbohydrate fermentation results. Blackening of the medium: Occurs in the presence of H_2. Gas production: Bubbles or cracks in the agar indicate the production of gas (formation of $CO_2$and H_2) (Kalyan 2018).

- **Simmons Citrate Test**

Single colonies were inoculated into simmons citrate agar (a mixture of salts, agar and bromothymol blue) and incubated for 24 h. If the reaction is positive, the medium will be seen blue. If the reaction is negative, the color will not be seen (MacWilliams 2009).

✓ Identification of Bacterial Isolates by VITEK-2 System

After the samples were cultured onto the culture media and incubated at 37 °C for 24 hours to obtain pure colonies for Vitek® 2 compact, (work was performed in al nukhbah laboratory) the VITEK system steps were used as according to instruction of its manufacturer:

Bacterial inoculum: bacterial cell suspension were placed in glass test tubes into a special rack while identification cards or cassette placed in apposite slot then the transfer tube transferred the suspension bacterial cell to identification cards to fill all tests wells, the cards contain up to 15 tests, after that the transfer tube was cutoff to seal the cards and to load into interior incubator at 37 °C for 24 h, the results read digitally on monitor connected to VITEK system apparatus (Wang and Song 2024).

Molecular Detection

Extraction of Genomic DNA from *E.coli*

The extraction DNA of *E.coli* by Genomic DNA Mini Kit according to FAVORGEN company as follows:

- The appropriate amount of bacterial cells were transferred to a 1.5 ml eppendorf tube and centrifuged at 14,000 xg for 1 min. the supernatant was discarded.
- The 200 µl of FATG Buffer was added and Incubated for 5 min at room temperature.
- The 200 µl of FABG Buffer was add to the sample and vortex for 5 seconds.
- It was incubated for 10 min at 70 °C until the sample lysate is clear.
- . The 200 µl of ethanol (96-100%) was added to the sample and vortex for 10 sec. Mix the sample well in the pipette if there is any precipitate formed.
- The FABG Column was putted to a Collection Tube, carefully transfer the sample mixture to FABG Column, Centrifuge at speed 14,000 x g for 1 min, then the Collection Tube was disposed and placed the FABG Column to a new Collection Tube.
- The 400 µl of W1 Buffer was added to the PABG Column and centrifuged it for: 30 sec at speed 14,000 x g. The filter was discarded and placed the FABG Column back to the new Collection Tube.
- The 600 µl of Wash Buffer was added to the FABG Column and centrifuged for 30 sec at speed 14,000 x g. The filter was discarded and placed the FABG Column back to a new Collection Tube.
- The centrifuge was performed for an additional 3 min at speed 14,000 x g to dry the column.
- The dry FABG Column was putted to a new 1.5 ml eppendorf tube.
- The 100 µl of Preheated Elution Buffer or TE was added to the membrane center of FABG Column.
- The FAGB Column was incubated at 37 °C for 10 min in an incubator.
- The centrifuge was run for 1 minute at full speed 14,000 x g to elute the DNA.
- The DNA fragment was stored at 4°C or -20°C

DNA Purity and Gel Electrophoresis

The DNA quality was examined using a normal 1% (W/V) agarose and 80 V at 35 min. Their Genomic amount and purity are assessed using a Nano-drop (1X) TBE buffer, using a 100 bp ladder as a molecular weight marker (Cat # D-1040, Bioneer, Daejeon, South Korea), the isolated DNA was used as a template for amplified a desired region of genes.

Thermocycler Technique

The gene of *16Sr*RNA was amplified using the PCR technique. This amplify universal primers (for word and reverse) of *16Sr*RNA was used by (Macrogen, Korea) as shown in table (1), suspended by dissolving the lyophilized depending with production instructed, stock solution (100 µl), working primer

(10 µl) prepared in dilution nuclease-free water, the working primer is prepared from stock suspension by take 10 µl from stock solution and add 90 µl of nuclease-free water (Dash, Rai *et al*. 2016).

Table (1): Primer used for molecular identification of pathogenic bacteria

Primer names	Primer Sequences (5'-3')	Product Size bp	Source of primer
Identification for bacteria Universal 16SrRNA			
***Universal 16Sr*RNA**	F-(5' -AGAGTTTGATCCTGGCTCAG-3')	1500 bp	(Loy, Lehner *et al.* 2002)
	R-(5' -GGTTACCTTGTTACGACTT-3')		

- **PCR Mixture**

Optimization of polymerase chain reaction was accomplished after several trails, thus the following mixture was according to information of (Pomega, PCR-Pre Mix-Kit).

Table (2): The PCR reaction mixture.

Component of mixture solution	Volume
Forward primer	2µL
Reverse primer	2µL
DNA template	5µL
Master mix	12.5µL
Deionized water	3.5µL

Table (3): PCR Thermocycler condaitions.

Primer	Temperature (ºC) / Time					Cycle number
	Initial Denaturation	Cycling condition			Final Extension	
		Denaturation	Annealing	Extension		
***16Sr*RNA**	95 ºC for 2 min	95 ºC for 30 sec	53 ºC for 30 sec	72 ºC for 2 min	72 ºC for 2 min	35

Identification of Isolates Based on *16Sr*RNA Sequence

Primers of *16Sr*RNA was used mainly to identify this same isolates. The RNA is an evolutionary marker that really is available throughout all pathogens and now has got to play a significant role in the evolution of microorganisms phylogenetics and classification. Bacterium are already characterized by

the common ancestry of their sequenced genomes, so any crude extract could be distinguished with great certainty if the nucleotide sequences of said type varieties indicating all the famous bacterial species were available (Nikunjkumar 2012).

Bacteriocin prodaction

Identification of bacteriocin-producing *Escherichia coli*

The main focus of the examination is the selection of bacteriocin-producing isolates. The isolates were investigated using the agar-well method (Thonda, Oludare *et al.* 2024), using the Mueller-Hinton agar or the Neutron agar. Zones of inhibition are reported in millimeters. Another type of *E.coli* and *Klebsiella* were used as references to investigate bacteriocin production. Briefly, agar plates were inoculated with bacterial isolates (*E. coli*) using the McFarland method under sterile conditions, and the holes (diameter = 8 mm) were filled with 100 µL of test samples and incubated at 37 ºC for 24 h. After incubation, the diameter of the growth inhibition zones was measured.

The overproduction of bacteriocin

Bacteria producing bacteriocin (colicin) were multiplied and stimulated by adding 20 ml of *E.coli* stored with Brain Heart Broth to 1L of Brain Heart Broth prepared with sterile conditions, then adding 0.001 mg of mitomycin-C to stimulate the bacteria to produce colicin (Charkhian, Soleimannezhadbari *et al*. 2024). The prepared medium was incubated in a shaking incubator to provide Good ventilation at a temperature of 37 ºC for 48 h. After incubation, we run centrifuge to the medium at a speed of 14,000 x g per second for 15 min, then discard the sediment and keep the filtrate at refrigeration temperature (Tarelli, Carminati *et al*. 1994).

Results and Discussion

Sample collection and bacterial Isolation

Forty clinical samples of bacteria were collected during the period from early August 2023 to the end of September 2023. They were collected in sterile cartilage from patients with diabetic foot ulcers, urinary tract infections, and wounds. All samples were immediately transferred to the laboratory for cultivation, where the culture results showed that 19 aerobic bacterial isolates were pure, compared to 31 isolates are culture negative. Negative results may be due to other factors, such as the presence of other anaerobic bacterial species, fungi, or others.

Identification of Bacterial Isolates

The initial bacteriological diagnosis, bio-chemical tests and Vitec2 compact system, in addition to molecular detection.

✓ **Initial bacteriological diagnosis**

The phenotypic characteristics of the bacteria colonies were appeared through their growth on culture agar medium, these characteristics are (shape, size, color, texture, smell and fermentation of sugar).

- ***Escherichia coli***

The results of culturing *E.coli* isolates on MacConkey agar showed that the colonies are spherical in shape and have a pink color due to the fermentation of lactose, and on Eosin methylene blue agar they are metallic sheen. These results are diagnostic for *E.coli* according to (Tille 2015). Similar results were shown according to (Abdul-hussein, Raheema *et al*. 2018). As shown in the Figure (1 A,B).

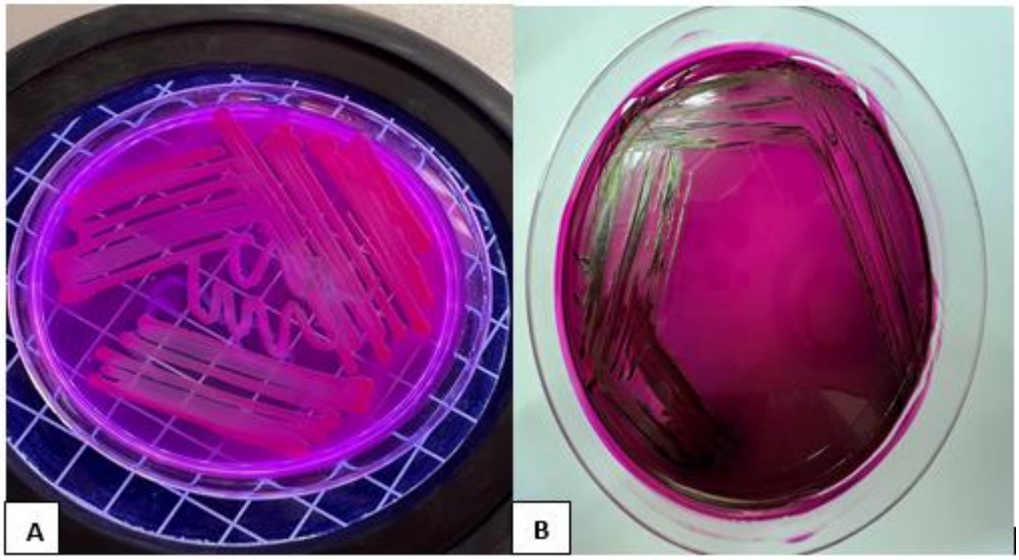

Figure (1): Show A- *E.coli* on MacConkey Agar Medium, B- *E.coli* on Eosin Methylene Blue Agar.

✓ **Microscopic Examination**

The results of microscopic examination of *E.coli* isolates showed that they were Gram-negative by staining them with the Gram stain, and they were diagnosed as pink-colored bacilli, as shown in Figure (2). These result are consistent with the study (Albadri, Noor *et al*. 2021).

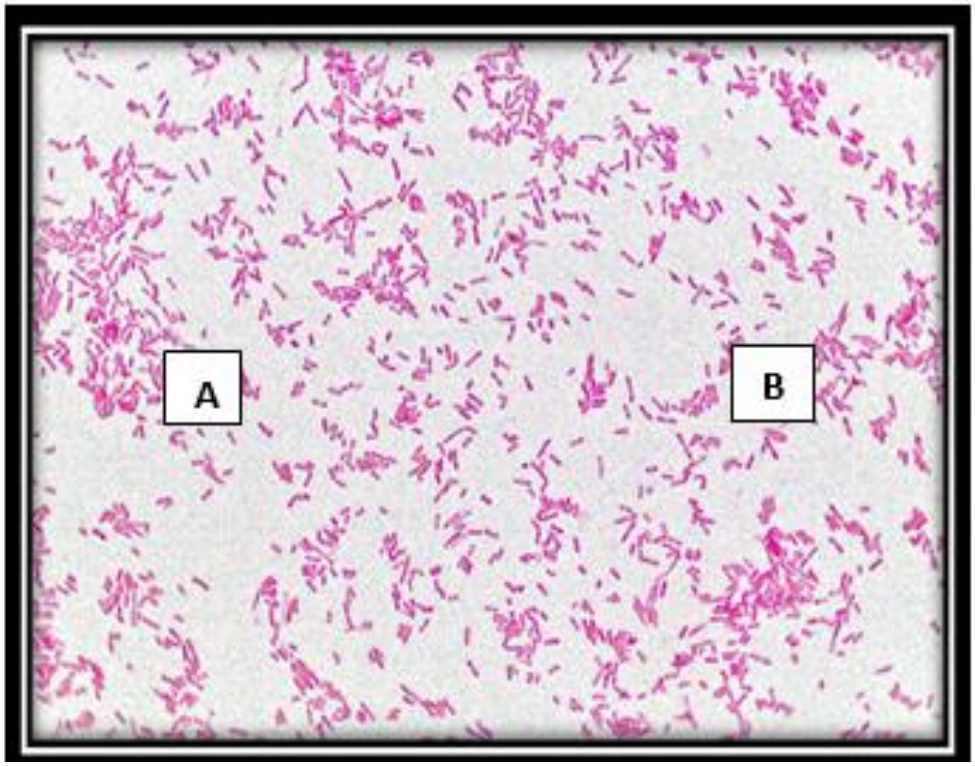

Figure (2): Microscopic Examination of *E.coli* under 100x showed (Gram negative bacilli).

✓ **Biochemical Tests**

Based on the working principle of each test, these tests were performed on *E.coli* isolates:-

The results of the chemical tests for *E.coli* showed that it was positive for the catalase test and Indole test.

While the results of the oxidase, urease, and Simon citrate tests showed negative results, TSI test showed Alk/A with H_2S as shown in the table (4). These similar results was recorded by (Al-Saeedi, Khajeh et al. 2019, Albadri, Noor *et al*. 2021).

Table (4): Shows The Biochemical Tests for *E.coli*.

Bacteria Isolation	Biochemical tests					
	Indole Test	Simmons citrate	Triple Sugar Iron	Urease Test	Oxidase Test	Catalase Test
E.coli	+	-	A/A with gas	-	-	+

(+) Positive, (-) Negative

✓ **Identification of Bacterial Isolates by Vitek2 Compact System**

The results of diagnosing the bacterial isolates with the vitek2 compact system showed that they were *E. coli* at a probability of 97%. This study is consistent with the results of (Abdul-hussein, Raheema *et al*. 2018). Index

Genotyping Identification of *E.coli*

Genomic DNA Extraction from *E.coli*

The clinically *E.coli* isolate was identified through DNA extraction according to the instructions of the genomic DNA mini kit of the (Korean FAVORGEN Company). The DNA was successfully extracted, as its purity is a good indicator of the extraction process and it does not contain any impurities that would hinder the process (Wilfinger, Mackey *et al*. 2006).

The results of DNA extraction showed good, as it was electrophoresis (1% agarose for 35 minutes at 80 volts) and then exposed to ultraviolet light. It was observed that the DNA appeared in the form of compact bands, as shown in the figure (3) . The results of the study are consistent with (Shahu, Vtyurina et al. 2024).

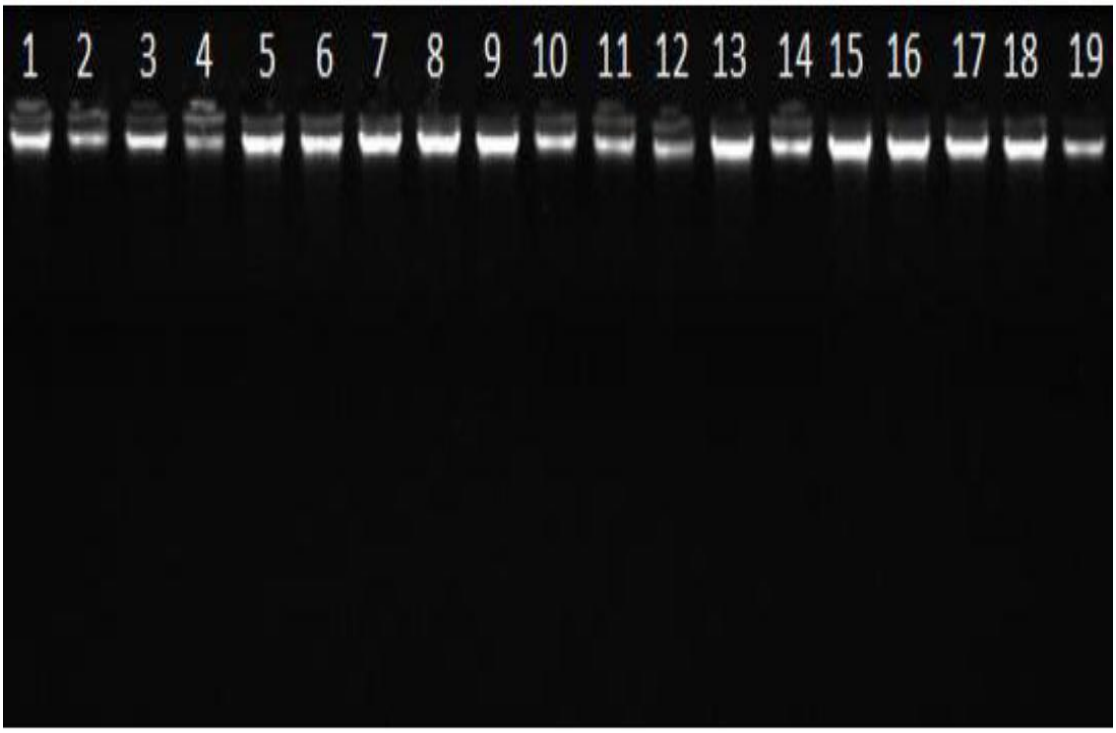

Figure (3): Integrity of genomic DNA extracted from representative samples. The DNA run on a 1% agarose gel at 80 voltages for 35 min, stained in a 500ml of 1X TBE buffer containing 0.7 µ of ethidium bromide, then visualized by a UV transilluminator.

Molecular Identification of *E.coli*

The *16Sr*RNA PCR product was obtained using PCR program as missioned in chapter three. The PCR product was visualized by agarose gel electrophoresis (Agarose 1.5%, 80 voltages for 1h). Using 1500 bp ladder as shown in figure (4)

The results of the current study are consistent with (Srinivasan, Karaoz *et al*. 2015) those identified the following bacterial isolates: *E.coli, K.pneumoniae , P.aeruginosa, S.aureus, P.mirabilis*.

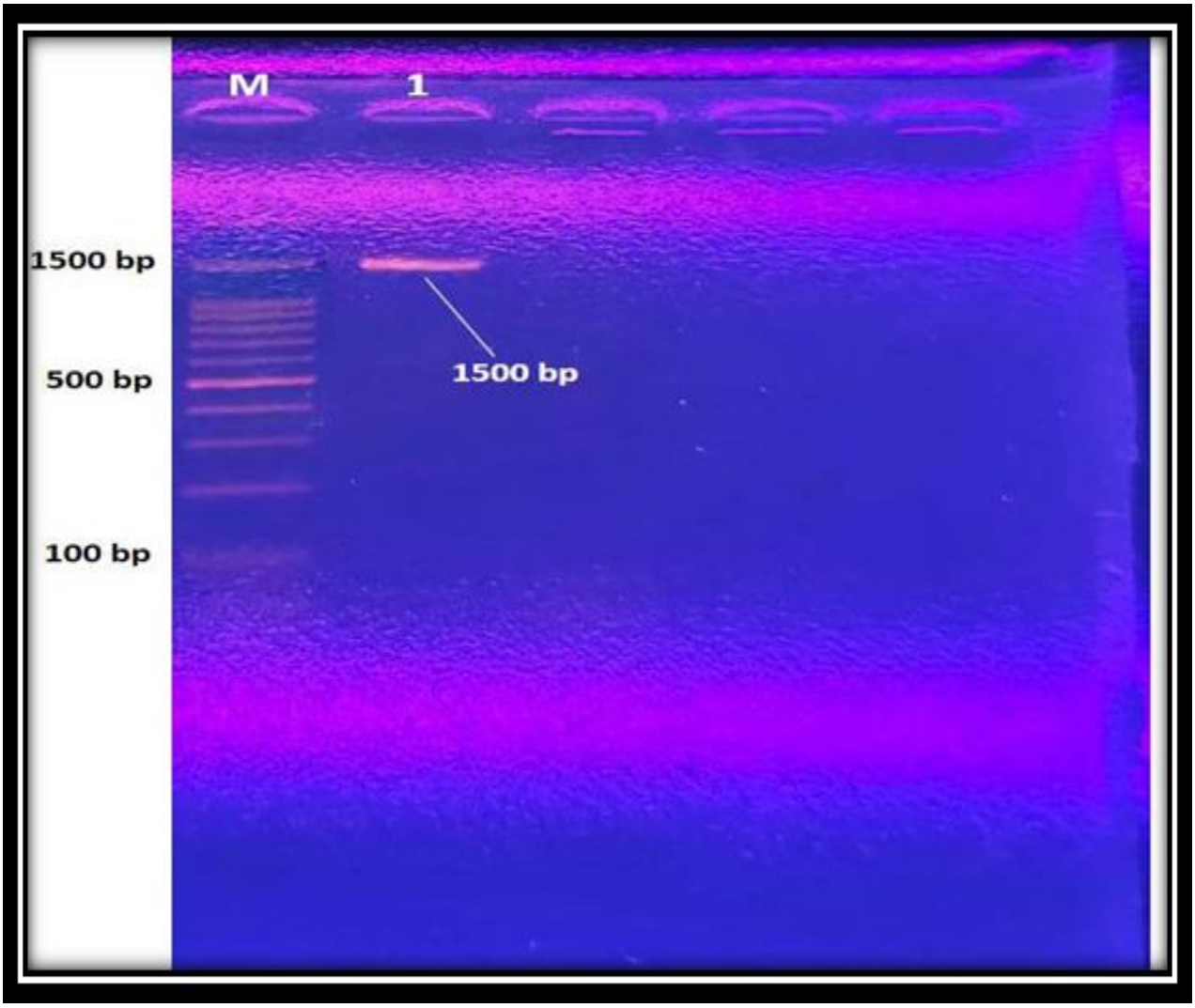

Figure (4): PCR product of an amplified *E.coli* isolate No. 19 with a 100 bp ladder . The PCR product was electrophoresis by a standard 1.5% (w/v) agarose gel that is pre-stained with ethidium bromide (0.7 µg/ml) in TBE buffer, using a 1500 bp ladder as a molecular weight marker at 80 voltages for one hour.

PCR product was transferred for DNA sequencing (Macrogen company). Sequence analysis was carried out by a BLAST program in NCBI where the *16Sr*RNA gene sequences of this *E.coli* isolate were aligned. The gene sequence result was received via the Internet and aligned with a NCBI database using the PLAST program, Accurate identification of bacteria is very important for any study. Molecular methodology is one of the best and most powerful methods used to identify and classify the bacteria used based on DNA sequences. The *E.coli* was identified and showed that it was 97% similar to other bacterial isolates in the GenBank database (NCBI), as in the figure (5). These study agree with the approach of (Al-Ouqaili, Khalaf *et al.* 2020). The sequence analyses of these isolate showed strong similarities with the representative isolates that were retrieved from NCBI database, and also determined the phylogenetic affiliation, according to nucleotide homology and phylogenetic analysis, as in the figure (6).

Figure (5): The *E.coli* was identified and showed that it was 97% similar to other bacterial isolates in the GenBank database (NCBI).

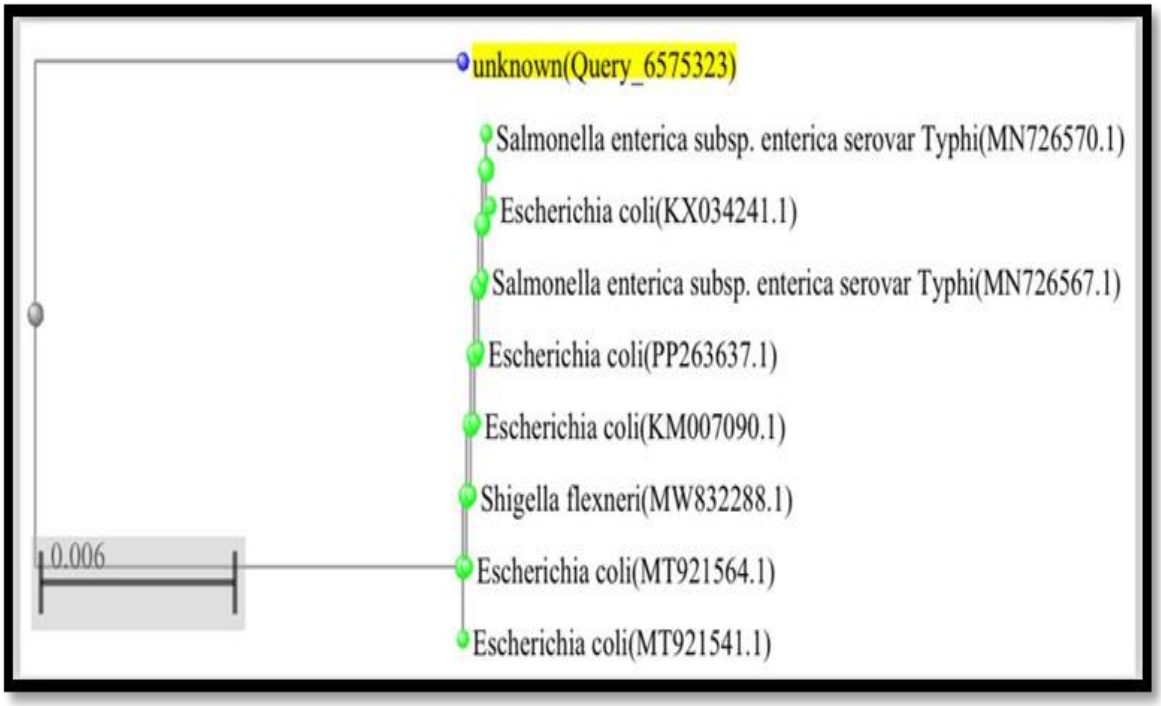

Figure (6): The phylogenetic tree of *E.coli*

Bacteriocin prodaction

Identification of bacteriocin producing *E.coli*

The production of bacteriocins from 40 *E.coli* isolates was investigated, and it was found that only one of the isolates showed inhibitory activity against other bacteria (*E.coli* from another genus and *Klebsiella*) according to the agar well diffusion method, where the diameters of the inhibition zones around the holes were measured in millimeters, as shown in the table (5) and figure (7).

From the above results, it is clear to us that the solid medium stimulates the antagonism between the two isolates and the production of bacteriocin and its spread in the medium, and this agrees with the results of (Al-Dulami 1999).

Table (5): Susceptibility of bacteriocin-producing *E.coli* against pathogenic isolates using the Agar Well Diffusion Method.

Local isolations	Activity against test bacterial isolation (diameter of the inhibition zone in millimeters (mm))	
	Other *E.coli*	*Klebsiella*
E.coli	21 mm	22 mm

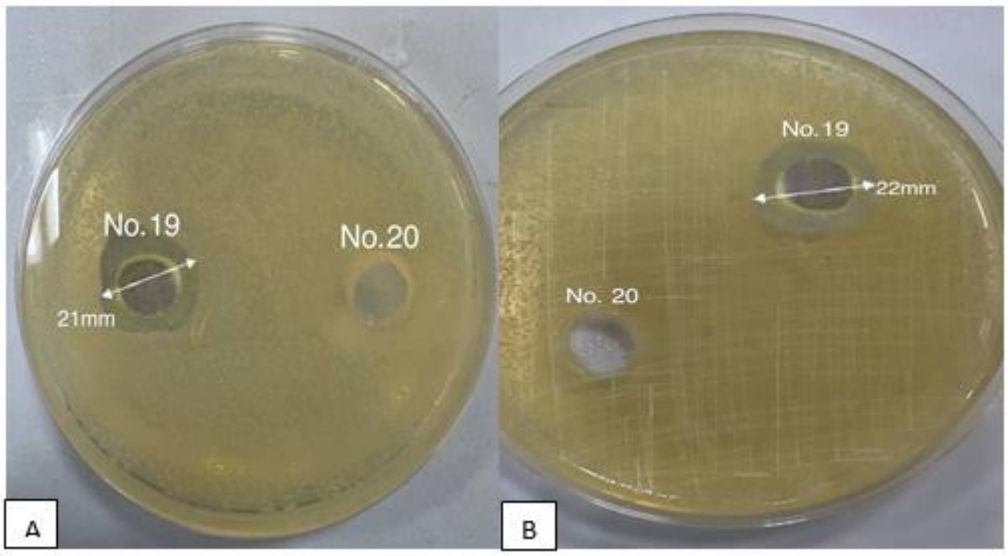

Figure (7): Inhibitory activity of bacteriocin produced from isolate *E.coli* No. 19 using the Agar Well Diffusion Method against the test bacteria A- *E.coli* of another genus, B- *Klebsiella.* (100µl of *E.coli* in the well)

The overproduction of bacteriocin

Bacteriocin biosynthesis is commonly seen during the stationary phase of microbial growth culture. Densely growing bacteria were obtained in Brain Heart Broth culture media by stimulating them to produce bacteriocins with mitomycin C after incubating them at a temperature of 37 ºC to 48 h and providing ideal conditions for the growth of these bacteria. Environmental conditions, including temperature, time, and essential nutrients, play an important role in the process of stimulating bacteriocin production.

This study agree with (Tarelli, Carminati *et al*. 1994), which explained the decreasing in bacteriocin activity after 48 h incubation may be by bacteriocin degradation due to culture proteases, or high temperature.

This study is not consistent with (Kang and Lee 2005) reported that the optimal production of bacteriocin was obtained at a temperature of 40 ºC.

Discussion:

To achieve the aim of the study, *E.coli* isolates were collected from different sources. The bacteria-producing bacteriocin was investigated by using the agar-well diffusion method. The Mueller-Hinton culture medium was used. different conditions control the produce of the bacteriocins isolates. Although it is a successful method, it is not without some defects, including the thickness of the agar layer, which may hinder the diffusion of the produced bacteriocin, and its effectiveness is not shown (Cabo, Murado *et al*. 1999). In general, the solid medium is much better than the liquid medium when investigating production, as it depends mainly on stimulating the antagonism between the two isolates (Cursino, Šmarda *et al*. 2002). In addition, the medium in which the screening test is conducted has a distinct and important role in showing the productive isolates and their indicator isolates, as the type, concentration, and depth of the agar layer are of great importance in this, and the density of the indicator isolate is taken into account, as it is preferable that it does not exceed 52-62 cells/ml. The bacteriocin produced, even in small quantities, can show lethal activity. On the other hand, microorganisms have a fundamental and major role in identifying bacteriocin-producing isolates, as there are many bacteria that can produce more than one type of bacteriocins, such as colicin, which gives them the ability to resist many types of other bacteria. The researcher Riley (Riley 2011) found that (70%) of the productive *E.coli* isolates have single resistance, while (30%) have joint resistance to three or more of them. Mitomycin-C was chosen to stimulate *E.coli* bacteria to produce colicin. to understand the mechanism of stimulation, the use of stimulating substances such as mitomycin-C, heat, antibiotics, etc., work to destroy the chromosomal DNA and stop all the vital activities of the bacterial cell, and immediately (SOS regulator genes) begin the repair process to save the cell at the last moment by stimulating many genes through special mechanisms to repair the error caused by the effect of that mutagenic substance (Mader, von Bronk et al. 2015). One of the genes that is stimulated is the Rec A gene, which in turn produces an enzyme protein Rec A Protease, and the latter works on special targets, which are proteins that produce chromosomal Lex A proteins. When these proteins are cut and inhibited by the aforementioned enzyme, the structural gene in the bacteriocin plasmid begins to work, as the process of gene multiplying begins, and the number of copies of this plasmid increases, thus increasing production. To avoid any error in the production process, the production-stimulating material must be added in appropriate quantities and under ideal conditions in terms of acidity, temperature, and the time required to incubate the bacteria and prepare them for production. About 2 grams of pure colicin were produced by isolating one out of 5 *E.coli* isolates. The purity of colicin and the absence of any other substance were confirmed by detecting it by the SDS-PAGE polyacrylamide method when a single band appears in the acrylamide gel. This is done by estimating the molecular weight of colicin by matching the molecular weight with the molecular weights of other standard proteins.

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