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Selenium Nano-particles bio-synthesized by pathogenic bacteria having antibiofilm activity analysed by fluorescent microscopy and Molecular detection of antibiotic resistance genes

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***Abstract*---**The purpose of this research was to study the biological activity of probiotic against MDR bacteria that isolated from diabetic foot infections. During the months of October 2020 to March 2021, a total of 65 specimens and investigations were collected. Specimens for diabetic foot infection were gathered from two hospitals in the province of AL-Najaf (AL-Sadder Medical City and AL-Hakeem Hospital). Swabs from diabetic foot infection patients were collected and sent to the lab, where they were streaked on MacConkey agar, a blood agar., around 40 (61.5%) exhibit positive bacterial growth culture, whereas 25 (38.5%) show negative findings for culturing. Negative growth might be due to other infection-causing agents such as anaerobic bacteria, fungi, and viruses [1]. Gram-negative bacteria had a high rate of 42 (64.4%) based on morphological and biochemical tests, with B. cepacia having a high percentage of 14 (33.3%) and A. baumanni having a high percentage of 12 (28.7%). K. pneumonia has 7 (16.6%), E. cloacae has 6 (14.4%), and no growth in 3 (7%) as shown in figure (4-2). while G +ve bacteria recorded 23 (35.4%), with S. aureus being the most isolated bacterium in this investigation with a percentage of 5.5 (23.9%), followed by S. epidermidis with a percentage of 3.5 (15.1%), as shown in figure. The first diagnosis of bacterial isolates was based on characteristics such as Gram stain, morphology, and biochemical testing. The automated VTEK-2 small

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equipment was utilized for the final identification, which included 64 biochemical tests on Gram-negative-ID and Gram-positive-ID cards. Isolated bacteria were tested against E. coli as an indicator strain for Selenium Nanoparticle production. The most effective isolate was chosen based on the colours that change from light to dark (Figure 4-5) and the biological effectiveness of Selenium NPS in E. coli against multidrug-resistant bacteria. Klebsiella pneumonia S6, Lactobacillus. helveticus S7 was the most effective isolate. Because each microbe has a unique metabolic process and enzyme activity, not all bacteria can manufacture NPs [2].

***Keywords*---**fluorescence analysis, Klebsiella pneumonia, resistance genes, bla-TEM, GES, bla- OXA.

Introduction

A diabetic foot is a lengthy or "chronic" complication of diabetes mellitus that develops as a result of peripheral artery disease (PAD) and/or sensory neuropathy in the feet. Diabetic foot syndrome refers to the occurrence of common diabetic foot problems such as infection, diabetic foot ulcer, and neuropathic osteoarthropathy. Antimicrobial-resistant (AMR) infections are caused by a microbe that is resistant to an antimicrobial agent. Many disease-fighting drugs lose their effectiveness microbes evolve resistance (Ali, 2018). AMR infections kill over 700,000 people per year across the world. By 2050 up to 10 000 000 people a year could die of antimicrobial resistance.[3] . Recent research concluded that molecular antibiotic resistance genes bla-TEM, GES, bla-OXA, would help predict antibiotic resistance in bacteria[4].

For producing various types of nanoparticles, a variety of physical, chemical, and biological processes are available, some of which are more costly, energy-intensive, and possibly harmful to the environment [5]. The synthesis of green nanoparticles is an interesting topic of nanoscience and nanotechnology. In contrast to damaging physical and chemical techniques, it comprises the development of clean, biocompatible, non-toxic, and ecologically acceptable nanoparticle manufacturing technologies. In the current scenario, diverse microorganisms have been used to synthesise nanoparticles with well-defined chemical compositions, sizes, and shapes[6].

Their applications in many cutting-edge technological sectors have been investigated This research examines recent advances in the production of a variety of nanoparticles using microorganisms. Diagnostic, treatment, pharmaceutical delivery, medical equipment coating, sterile gauze, therapeutic fabrics, contraceptive devices, antifungal, anti-inflammatory, and other uses have all used nanoparticles. The future of bacteria-based nanoparticle production has also been discussed[7]. Strong photocurrent, catalytic performance in hydrolysis and oxidizing reactions, and strong piezoresistive, thermal, and photonic reactions are all characteristics of selenium (Se) NPs[8]. Selenium nanoparticles have employed in a number of biological applications as medication delivery systems and anticancer agents[9].

Materials and Methods

Preparation of Culture Media

The media prepared according to the manufacturer's step instructions. In a microwave oven, the ingredients are entirely dissolved in clean water. The media has been sanitized. After autoclaving for 15 minutes at 121°C, the samples were put into sanitized Petri dishes. Within a laminar hood for agar media and sterilized screw tubes for broth media to maintain sterility for 24 hours at 37°C [3].

Molecular detection of antibiotic resistance genes (bla-TEM, GES, bla- OXA) in tested Bacteria

(Macrogen/Korea) generated these primers (Table 1-1). Which were dissolved in sterile deionized water. The standard solutions (100 µl) created by adding, ddH2O to vial holding the lyophilized primers, and the working solution (10 µl) was made by combining (10 µl) of the stock primers with (90 µl) of ddH2O. Both the stock and the working solution were kept in -20°C.

Table 1
The sequence of Primer that used in present study

Primer	Direction	Sequence (5-3)	Product
TEM	Forward	TCAACATTTTCG TGTCGCCC	766
	Reverse	AACTACGATACG GGAGGGCT	
OXA	Forward	AGATCCTTGACC CGCAGTTG	928
	Reverse	CGCCGTCCCAT CGAAAAATC	
GES	Forward	TCACTCTGCATA TGCGTCGG	692
	Reverse	ACTTGACCGACA GAGGCAAC	

PCR Reaction Mixture

To amplify DNA target fragments (TEM, GES, OXA) genes, the polymerase chain reaction (PCR) was utilized (Table 2-2).

Table 2
Contents of the Reaction Mixture

Contents of reaction mixture	Volume (µl)
Master Mix	5
Template DNA	4
Forward primer (10 µm)	2
Reverse primer (10 µm)	2
Nuclease free water	Up to 20

Polymerase Chain Reaction (PCR) Condition

Specific primers were employed in conventional PCR to amplify a target DNA fragment. To create PCR product, PCR primarily comprises three steps of repeating cycles. (Denaturation, annealing, and elongation) (amplicon). The following table lists the PCR thermal cycling conditions (2-1). Electrophoresis in (1.5) per cent (w/v) agarose gel with (1) X TBE buffer and stain with Red safe dye that used to quantify the size of PCR products (5 L). A size 100 bp DNA ladder (Intronbio/Korea) used to determine the size of the production**.**

Table 3
PCR conditioned for amplification

Step	Temperature (°C)	Time	No. of cycles
1- Initial Denaturation	95	5 Minutes	1
2-Denaturation	95	30 Second	35
3-Annealing	56 (bla-*TEM*, *bla- OXA*) 58 (*GES)*	45 Second	
4-Extension	72	60 Second	
5-Final extension	72	5 Minutes	1
Storage	4	Hold	1

Determination of the purity and concentration of DNA

Optical density of dsDNA measured in 260 nm, to quantify its concentration spectrophotometrically. Ratio of OD260/OD280, which is in the range of (1.8± 0.2) for pure DNA, indicates the quality of the DNA solution [4].

Agarose Gel Electrophoresis

Separating of the DNA fragments on agarose gel electrophoresis is the most common successful methods. The quantity of agarose in the gel varies depending on size of DNA fragmentation that will be separated, and can range from (0.5 to percent). Following extraction, a 0.7 percent gel was used to separate genomic DNA (5-10 kb), whereas 1.5 percent -2 percent gels were employed to achieve excellent resolution for microscopic PCR product fragments (0.2-1 kb). However, to the indicated weight of agarose, 100 mL of 1×TBE buffer was added, which was then boiled in microwave tile it turned clear. After the agarose was cooled to 50-55°C, 5 µl of simply safe dye (10 mg/ml) added to 100 ml of melted agarose gel for achieve a final concentration of 0.5 µg/ml after the agarose was cooled to 50-55°C. (Rusell and Sambrook, 2001). Agarose then placed into the specific gel tray, and the comb was appropriately inserted before drying. The samples were placed into their wells on the gel, while the marker was injected into one well. The poles were connected appropriately, and the run was completed according to gel per cent and the size (The agarose gel electrophoresis time is 45 minutes, for the genomic DNA and for (PCR) DNA sequencing of 16sRNA of tested isolate is 1 h and 30 min).

Extraction of DNA for Sequencing

Before the wash buffer was used for the first time, absolute ethanol was added. The following was the gel extraction DNA batch processing. An agarose gel slice was used to extract the required DNA fragments, and the excess agarose removed to make the gel slice smaller. Up to 300 mg of gel slice placed in (1.5 ml) micro-centrifuge tube. The material was vortexed combined with 500 µl of DF Buffer. The DF column placed in a (2ml) collection tube, and 800 µl of the mixture from the step 1 transferred to DF Column and centrifuged in 16000 rpm for 30 sec. The DF column then reinstalled into the (2 ml) collection tube after the flow through discarded. After filling DF Column with 600 µl of washing buffer, it was let to settle for one min. After centrifuging for 30 sec at 16000 rpm, the flow-through was discarded. Flow through then removed, and DF column was reinserted to the (2ml) tube collecting. Column matrix centrifuged for 3 min at 16000 rpm to dry it. The dried DF Column moved to a new (1.5 ml) micro-centrifuge tube, and the column matrix was eluted with 50 µl of elution buffer. The elution buffer will be consumed by the matrix for 2 minutes. The pure DNA eluted by centrifugation at 16000 rpm for 2 min[5].

Bacterial Biofilm by Fluorescence Microscopy Analysis

Bacterial biofilms are layered forms on the substrate surface that are three-dimensional and enclosed in hydrated extracellular polymerics substance (EPS). Microbial EPS is a biopolymer made composed of polysaccharides, proteins, and nucleic acids[6]. The formation of a stable arrangement of bacteria in a biofilm is aided by EPS. High molecular weight compounds, such as polysaccharide-protein and amphiphilic polymers, make up the majority of EPS. The researchers used fluorescence and confocal laser scanning microscopy to look at how the microorganisms in the research developed biofilms fluorescent dyes specific to biofilm components such as EPS, living cells, and dead cells that used to stained

biofilm structure, which including EPS, living cells, and dead cells in the case of biofilm[7] . Goal of this project is using a fluorescence microscope to produce and examine bacterial biofilms dyed with acridine oranges. Chapter Two Literature Review 39 The fluorescence intensity of the biofilm photos will be calculated and a 3D structure of the biofilm will be drawn using the Image J program[8]. However, to gain a detailed picture of the isolates' biofilm architecture, it must be dyed using the appropriate colour. The most common pigment used for this purpose is acridine orange AO which binds to (nucleic acid), propidium's iodide which attaches to the (DNA), and tetramethyl rhodamine isothiocyanate concanavalin A which bind to DNA (binds to glycoprotein). Imaging captured during the discoloration procedure followed by using fluorescence microscopy must be examined to provide a pure image and a comparison of biofilm growth by the bacteria[9].

Results and Discussions

Demographic Profile of Patients with Diabetic Foot Ulcer

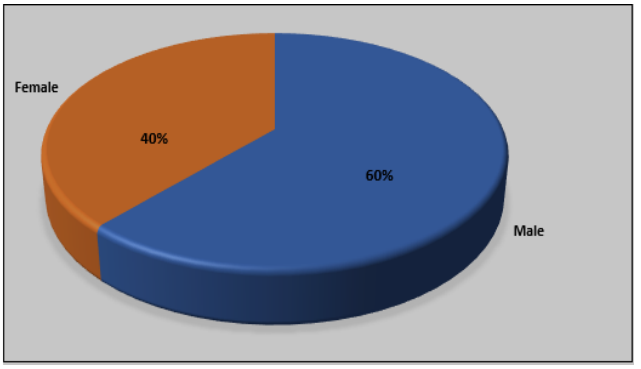
During September and December 2021, 65 specimens from diabetic foot sores of probable patients were studied. According to the findings, clinical specimens were 65 dispersed according to the patient's age range of (20-70) years old.
The lowest incidence was 6.1% and 1.6% in the 20-30 and over-60 age groups, respectively, while the greatest incidence was 38.5% in the 51-60 age group, as seen in table (4-1).

Table 3.1
The patient's distribution according to age groups and gender

Gender	Age group No. (%)					
	20-30	31-40	41-50	51-60	61-70	total
Female	1 (1.5)	4 (6.1)	10 (15.3)	11 (17)	0 (0)	26
Male	3 (4.6)	9 (13.9)	12 (18.5)	14 (21.5)	1 (1.5)	39
Total	4 (6.1)	13 (20)	22 (33.8)	25 (38.5)	1 (1.5)	65

Figure 1 shows that out of 65 patients, 39 (60%) were male and 26 (40%) were female (4-1). Increasing the number of infected males over females, comparable to the findings of Bansal et al., (2008), who found that male was infected at a higher rate than female (78.64 per cent versus 21.36 per cent) among 270 diabetic patients with foot infections (180 males and 90 females). With an age range of 36 to 75 years, the male-to-female ratio was 2: 1, with males accounting for 72.2 per cent, according to[10]. Previous research has found that males are 2 times more sensitive than females, which is consistent with the current findings[10] . The study's findings of male patients outnumbering female patients can be explained

by the fact that males are exposed to more external environmental influences in our nation. The male preponderance in DFU may be due to gender-related disparities in living styles and professional activities that need the feet to withstand higher pressure as a result of labour, increased outdoor work, and poor compliance with foot care procedures compared to females. Greater oestrogen receptors in females boost healing wounds, which operate as endogenous enhancers of the healingocess, but increased levels of androgen in males were deemed bad for wound healing because androgenic species reduce dermis repair[11].



Molecular detection of antibiotic resistance genes

The DNA of (*B. Specie, A. baumanni, E. cloaca, and S. aureus)* were amplified by Polymerase chain reaction (PCR), using specific primers. It has been shown that the polymerase chain reaction (PCR) products for bla- TEM gene with the single band (766 bp). GES gene with the single band (692 bp), a bla-OXA gene with the single band (928 bp) PCR products were observed as seen in figure (4-4). 4 Molecular detection of antibiotic resistance genes The DNA of (B. cepacia, A. baumanni, E. cloaca, and S. aureus) were amplified using (PCR) technology, by using specific primer. It has been shown that the polymerase chain reaction (PCR) products for bla- TEM gene with the single band (766 bp). GES gene with the single band (692 bp), a bla OXA gene with the single band (928 bp) PCR products were observed as seen in Figure

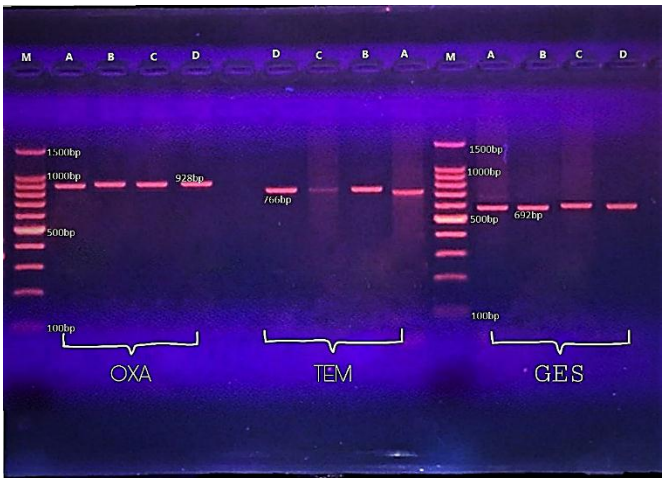


Figure:(3-1) Agarose Gel- Electrophoresis of TEM (766bp), GES (692bp), and OXA (928bp) products. A: S. aureus, B: A. baumanni, C: E. cloacae, D: B. cepacia, M: (DNA marker size (100bp), at 1.5% Agarose gel at 85 vol for 45 minutes

The result of molecular detection of antimicrobials antibiotics resistance genes positive for (bla-TEM, GES, bla-OXA) genes. This result agrees with[12] who found bla-TEM in B. cepacia and K. pneumonia. It has also been detected in other species of Gram-negative bacteria such as A. baumanni. bla-OXA and GES were found in all species, E. cloacae S. aureus, A. baumanni and B. cepacia. Plasmid-encoding β-lactamases are widely identified in S. aureus and A. baumanni also found in additional B. cepacia and E. cloacae bacteria, bla-TEM, bla-OXA treatment isolates [13]. Chapter Four Results & Discussions 80 Other bacterial species have been shown to have GES. Extended-spectrum beta-lactam medicines like Penicillin and Cephalosporins can be hydrolysed by these enzymes. The creation of extended-spectrum beta-lactamases (ESBLs) has resulted in bacterial resistance[14] .

Fluorescence Analysis of Bacterial Biofilm and Image Analysis of Selenium NPS

In a fluorescence microscope, fluorescence is employed to take pictures. The fluorescently coloured biofilm is exposed to the light of a certain wavelength, which is absorbed by the fluorophores and causes them to produce longer light of wavelengths. Which is differ from absorbed light[15]. AO (Acridine orange) a fluorescent pigment attaches to DNA specifically. It can pass through it. When combined with DNA, it exhibits an extreme excitation of 502 nm and a higher emission of 525 nm (green). When combined with RNA, however, the greatest illumination changes to 460 nm and the maximum emission to 650 nm (red)[16]. In 5 ml BHI broth, culture a loop of pathogenic bacteria (*B. cepacia*) incubated at 37 °C, overnight. Dilute preceding culture in BHI broth to 1:100. (For example, culture 1 mL in 99 mL BHI broth.) Place 5 ml of the above-diluted culture on a Petri-plate with a glass slide such that half of the slide is immersed in the medium to create a biofilm at liquid air interphase.

For the generate a submerged of biofilm, move 15 ml of above-diluted culture to a Petri-plate with a glass slide that is completely immersed in the media. Under static circumstances, incubate for 48 hours at 37°C. After proper incubation, lift the glass slide and rinse it 2–3 times with PBS, carefully vertexing to eliminate planktonic cells[17].Stain the slides for 5 minutes in the dark with a 0.02 per cent AO aqueous solution. Rinse well with 1X PBS, dry, and cover the discoloured area with a coverslip. Examine the changes with a fluorescent microscope and analysis of image-by-IMAGE J software, as shown in figure (3)

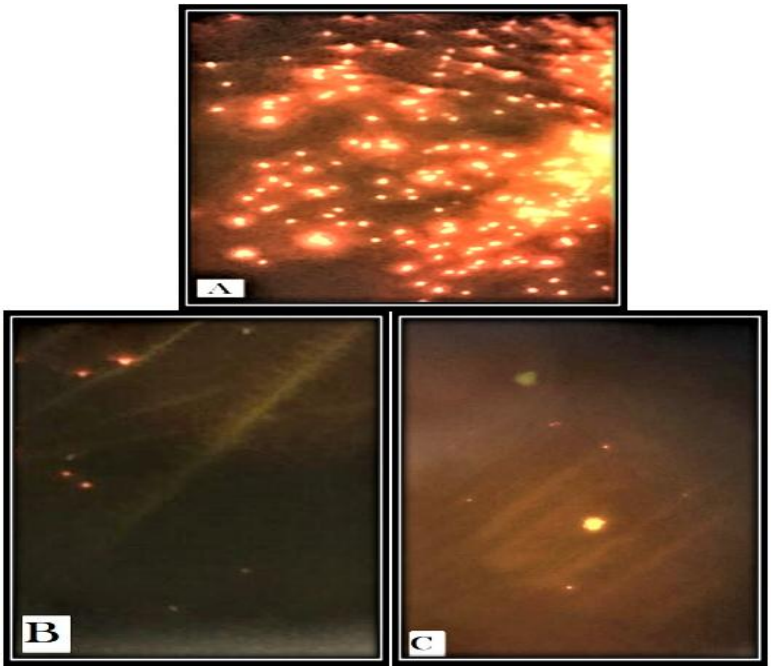


Figure (3-2): Selenium NPS with *B. cepacia* analysis by fluorescent microscope. A: *B. cepacia* only, B: *B. cepacia* with Selenium NPS synthesized by *K. Pneumoniae*

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