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Molecular and Anti Biofilm Study of Biosynthesized Silver Nanoparticles Mediated by *Lactobacillusplantarum* against *Klebsiellapneumoniae* Isolated from Urinary Tract Infaction

Fareed Kadhem Hamza[1], Qasim Najim Thewaini[2], Wejdan Ridha Taj-Aldeen[3]

1M.Sc., Student, Biotechnology Department-College of Biotechnology/University of Al-Qasim Green, 2Prof. Dr., Biotechnology Department-College of Biotechnology/University of Al-Qasim Green, 3Prof. Dr., Biology Department**-.College of Science/University of Babylon**

ABSTRACT

The isolate of *Lactobacillusplantarum.* was obtained from the dairy products. These isolates were cultured on De man rogosa sharpe broth and agar for 24 h at 37°C.Based on the biochemical tests, these isolates identified as *Lactobacillus plantarum*. and the fermentation test for carbohydrates distinguished the *Lactobacillus.plantarum.*, this isolate was diagnosed by biochemicaltests and using Vitek2 system as *Lactobacillus plantarum*, The method of biosynthesis of nanoparticles is gaining an extremely significant field due to its economic and environmental advantages compared to the chemical and physical methods of synthesis.
The goal of the current study is to use Lactobacillus Plantarum to biosynthesize silver nanoparticles. Ag NPs were biosynthesized by adding silver nitrate (AgNO3) to the cell-free supernatant per Lactobacillus Plantarum at a concentration (5 mM) to be used as a precursor for the synthesis of Ag NPs. The color shift of AgNPs from yellow to reddish-brown was the first indication of biosynthesis. UV-visible spectrophotometry was used to characterize biosynthetic nanoparticles, and the maximum absorption peak of the nanoparticles (410 nm) was observed. The size and distribution of nanoparticles were determined using scanning electron microscopy (SEM), and the shape was circular, homogeneous, and the size varied from (30-100nm). The occurrence of Ag was investigated using Energy Dispersive x-ray Spectroscopy (EDS). Antibacterial activity of biosynthesized AgNPs against multidrug-resistant bacteria (MDR) of both gram-positive and gram-negative bacteria, as well as biofilm formation (Between September 2020 and March 2021, a total of 15 Klebsiella pneumonia isolates were collected from clinical samples of urinary tract infection. The identification of Klebsiella pneumonia isolates was established using morphological, cultural, and biochemical tests, and then confirmed using the Vitek -2 system. The numbers of obtained bacterial isolates according to site of infection wer (15) isolates urinary tract infection. regarding antibiotic susceptibility testing the present study demonstrate that all isolates (100%) were resistance to Ceftazidime, and Ceefpime (100%) respectively , whereas the lowest minocycline 26,67% du to misuse of antibiotics. The present study demonstrated the occurrence of some virulence factors such as capsule in all isolates (100%), biofilm formation was recorded in 10 isolates,. The molecular detection of some virulence genes (*wbbm, mrk*D and *wzm*) was done by conventional PCR technique. It was found that high occurrence.

The goal of the current study was to investigate the formation of biofilm to Klebsiella pneumonia and the relationship between biofilm formation and the presence of (wbbm, mrkD and wzm) genes of K. pneumonia isolates respectively .

Keywords: *AgNPs, , Klebsiellapneumonia* , capsule, biofilmformation, *wbbm*, *mrk*Dand *wzm*.

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1.Introduction

Nanotechnology is the analysis and modification of matter at the atomic and molecular scales on a scale of one billionth of a meter (i.e., 10-9m=1nm). The size of a nano product can vary from 1 to 100 nanometers (Rajesha S. et al., 2015). Silver nanoparticles (AgNPs) have gotten a lot of recognition because of their activity of antimicrobial and ability to resist the formation of the biofilm, just as well their special physical, chemical, and biological properties and applications in electronics, optics, and medicine (Ansari et al . 2014). For synthesizing different kinds of nanoparticles, there are physical, mechanical, biological, and hybrid approaches, as well as more advanced, energy-intensive, and potentially dangerous to the environment physical and chemical methods (Liu et al., 2011).

The most crucial element in expanding nanoparticles' biomedical applications is to develop effective, non-toxic, and environmentally friendly methods for synthesis. The use of microorganisms to synthesize nanoparticles is one option for achieving this aim (Gade et al., 2008). The extracellular synthesis of silver nanoparticles utilizing Lactobacillus bacteria, as well as the discovery of new efficient antimicrobial agents to overcome several tolerance of microorganisms to antibiotics, tend to be low-cost and environmentally secure (Chaudhari et al., 2012) (Franci et al., 2015).
In 1933, Henrici made the first known discovery about biofilm when he noticed have been water bacteria do not free-floating but grow on submerged surfaces (Toole et al., 2000). Biofilm is made up of multifaceted cell clusters embedded in an extracellular polysaccharide matrix that allows microorganisms to adhere to biomedical surfaces while also shielding them from the immune system and antimicrobial therapy (O Gara et al., 2001). The expression of a polysaccharide intracellular adhesive regulates biofilm formation. Biofilms are notoriously difficult to extract, and they are frequently resistant to systemic antibiotic treatment, necessitating the disposal of contaminated devices (Schwank et al., 1998) (Souli et al., 1998). Biofilm organisms have built-in antibiotic resistance.

Nanotechnology can provide a way to infiltrate such biofilms and minimize the formation of the biofilm by using "neofunctionalization surface techniques."
Nanotechnology has the potential to prevent life-threatening biofilms from forming over medical devices. Silver was once known to be an antimicrobial. AgNPs hydrogel hybrids of various sizes of AgNPs have recently been demonstrated to be effective antibacterial agents (Mohan et al.,2007). Saxena et al. discovered have AgNPs immobilized in propylene-based sutures have antibacterial activity against *Klebsiella pneumonia.*
Infections caused by microbial biofilm formation continue to be a significant concern to patients all over the world. Wound infections are especially troublesome (Percival et al., 2008), with chronic wounds like the foot, leg, and pressure ulcers being especially susceptible to infections of the biofilm (James et al.,2010). To destroy or remove biofilms, antimicrobials should infiltrate the polysaccharide matrix and enter the microbial cells. The advancement of AgNPs as a new generation of antimicrobial agents might be an appealing and cost-effective strategy for combating Gram-negative bacteria drug resistance. Using the Klebsiella pneumoniae tube system and the Congo red agar (CRA) method, the researchers investigated the anti-biofilm capacity of AgNP biofilms.

MATERIALS AND METHODS

Culture of *Lactobacillus*

The isolates of *Lactobacillus.plantarum.* was obtained from the dairy products which were stored in the Advanced Microbiology Laboratory /University of Babylon. this isolate was

diagnosed by using biochemical tests and Vitek2 system
(*Lactobacillus.plantarum*) was utilized as a source of *Lactobacillus*. De Man Rogase and Sharp broth (MRS broth) wasinoculated with *Lactobacillus.plantarum* and incubated under anaerobic condition using anaerobic jar with anaerobic gas back system at 37°C for 48 hrs. Colonies were captured and affirmed as Lactobacillus based on morphological and biochemical assays (Holt et al., 1994). The second activation was worked from the first activation at 37°C for 24 hrs (Gurunathan et al.,2009).

Ag Nanoparticles' Biosynthesis by *Lactobacillus plantarum*

The pure culture of Lactobacillus Plantarum (5 mL) was inoculated in a flask that contains De man Ragosa Sharpe broth (MRS) and incubated at 37 ° C for 24 hours at 100 rpm. After the incubation period, the centrifugation was done at 5,000 rpm for 25 minutes. Then the supernatant was taken. The pH of the supernatant was regulated by 0.4 M NaOH to delay the transformation process (the pH of the supernatant was acidic 4.4 to be neutral, NaOH was added to reach a pH of 7 to eliminate the effect of organic acids). Aqueous (Ag NPs.7H2O) 0.85 g. 0.005 M dissolved in 1000 ml of distilled water was added to 250 ml of the supernatant and then heated by a water bath at 85 ° C for 5-10 minutes. The transition mechanism is shown by the black deposition at the bottom of the flask. The flask was then incubated for 12 hours at 37 degrees Celsius. The ions clump together at the bottom of the flask. The product was centrifuged at 10,000 rpm for 20 minutes to extract the black precipitate. It was washed three times with deionized water to extract pure materials, then dried for four hours at 60 degrees Celsius in an oven with hot air, as shown in Figure (1). (Ishwakarma et al., 2018).

Characterization of silver nanoparticles

Analysis by Scanning electron microscope (SEM)

In the unit of the electron microscope, the Faculty of the Science / University of Kufa, a scanning electron microscope (S50; FEI assay) was used to characterize the morphology and size of the nanoparticles. Prepare the slides by placing a small drop of nanoparticle suspension for biosynthesis on the left slide, drying it, and then analyzing it with a scanning electron microscope (SEM). The microscope operates with a 5-10 kV accelerating voltage , various magnifications, low vacuum, spot size 4, and working distances of 5-10 mm.

The analysis of the Energy dispersive x-ray spectroscopy (EDS)

The electron microscope unit at the Faculty of Science/ Kufa University used a Bruker EDS attached to an SEM to perform an elemental study of single particles. This study was conducted with a 10 kV accelerating voltage, a spot size of 5, and working distances of 10mm to detect the existence of element nanoparticles (Sarvamangala et al., 2013).

RESULT

Biosynthesis of Silver nanoparticles

Lactobacillus Plantarum demonstrated its ability in extracellular biosynthesis of silver nanoparticles (AgNPs) utilizing cell-free supernatant and silver nitrate (AgNO3) as a precursor to the synthesis of AgNPs (5M) added to the cell-free supernatant of Lactobacillus Plantarum by shaking the 24h incubation at 37 ° Celsius, Lactobacillus Plantarum possesses the ability to change the color of the reaction mixture from yellow to reddish-brown (Fig.1) that is an indicator of the biosynthesis of AgNPs by Lactobacillus plantarum.

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Figure(1):Biosynthesis of Ag NPs by *Lactobacillus plantarum*.
A- Culture suspension of *L. plantarum* on MRS broth (24 h incubation). B- Addition of AgNPs to the suspension then 24 h incubation.

The X-Ray Diffraction (XRD)

Figure (2) demonstrates X-ray diffraction patterns of Ag NPs composite with L. Plantarum. XRD (hexagonal phase) analysis shows have the synthesized nanoparticles were crystalline and pure in nature. The peaks at 2θ = 38.01, 45.28, 56.41, 66.12 and 75.03 were consecutive lines of spherical Ag-O nanoparticles, respectively.

The average particle size of Ag NPs was determined by applying Scherrer's equation, as shown in Equation (2) for peak reflection 1 at theta using full-width half-maximum (FWHM) results reported by Ashokkumar and Muthukumaran, (2014); Chang et al. Al, (2015).

$$\Delta(2\theta).cos(\theta), k = 0.9, \lambda = 0.15418nm, 2\theta = 38,$$
$$D = 0.9\lambda k\ /$$

$cos\ 19 = 0.945$D = $\frac{k\lambda}{Bcos\theta}$ ⇨

D = 8.4278 nm

The maximum measured diameter of a particle is 8.4278 nm, which is a line extension of the diffraction peaks, suggesting that the composites are nanometer-sized. The obtained nanoparticles have a very low synthesis; this might be due to the biological synthesis process used in the preparation of the nanoparticles.

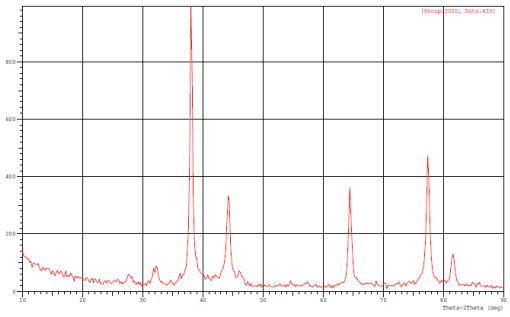

Fig. 2. Figure (2): XRD analysis for ag NPs biosynthesized by *L. plantarum*..

Atomic Force Microscope (AFM)

Atomic Force Microscope analysis of synthesized AgNPs was carried out to assess their morphology (outer surface) and size range ,as in figure (3) two-dimensional photo of a section of the surface of AgNPs with large molecular clusters up to 20 nm of AgNPs. The 2-D and 3-D images of AFM, figures (3, 4), showed that most of the nanoparticles are spherical

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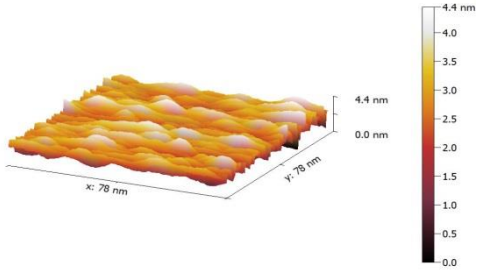
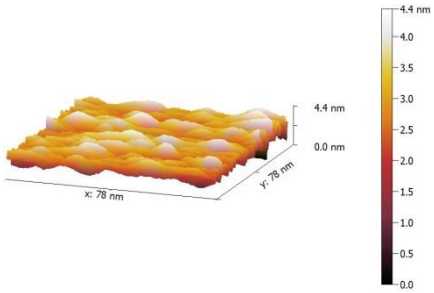
in shape and some of the agglomerations were present in the background of the nanoparticles. AFM analyses reached that obtained nanoparticles were in a hexagonal, polydispersed, nearly spherical in shape, these results were compatible with the study of(Nobel *et.al.*,2019). AgNPs on freshly cleaned

Si substrates were imaged by AFM (Figure 4). Nanoparticles ' lateral sizes differed from image to image due to the varying shape of the tips. Moreover,

20-140 nm has been found to be relatively constant in height ,this result was compatible with the study done by Monica *et.al*.,(2014) .



Figure(3)Two dimensional image of Ag NPs biosynthesized by *Lactobacillus plantaru.*



Figure(4) Three dimensional image of Ag NPs biosynthesized by*Lactobacillus plantarum.*

SEM analysis of nanoparticles

A scanning electron microscope (SEM) was utilized to characterize the shape, size, and distribution of the nanoparticles. After biosynthesis of silver nanoparticles utilizing cell-free supernatant of each Lactobacillus Plantarum. The results demonstrated the presence of homogeneous well-dispersed nanoparticles with a diameter (1-100 nm) of the silver nanoparticles, with different shapes, most of them in a spherical shape (5). The concentration of AgNO3 added to the cell-free supernatant also had an influence on the characterization of the nanoparticles formed, and 5 mM AgNO3 was the best concentration in the biosynthesis of AgNPs compared to the other concentrations..

EDS analysis of nanoparticles

The element occurrence was quantified by energy-dispersive X-ray spectroscopy (EDS) by observing the optical absorption peaks of the silver and titanium elements. The presence of the silver element indicates a decrease in silver ions in the reaction mixture by the Lactobacillus supernatant. In the spot profile mode, the EDS spectrum showed strong signals from Ag atoms, intermediate signals from oxygen, and weaker signals from other atoms. The weight percentage of primary components of AgNPs formed by Lactobacillus was 79.77%

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silver, 8.37% carbon, 6.39% chlorine, and 5.48%, (Fig.6). A peak of AgNPs was detected at 3Kev and is an atypical uptake of metallic AgNPs. Depending on the characterization of nanoparticles by SEM and EDS. The shape, size, distribution, and presence of metallic nanoparticles were determined and as a result AgNO3 (5 mmol) was used for further study.

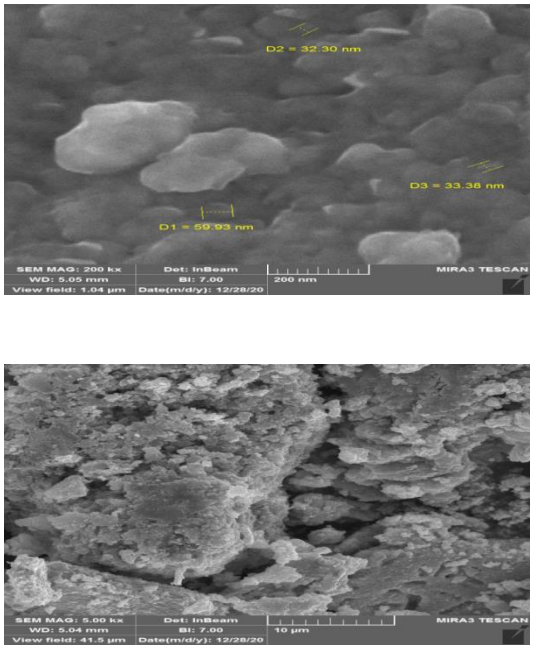
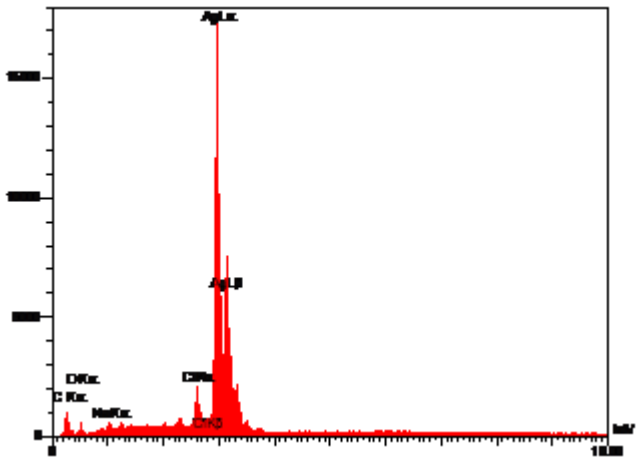


Figure (5):FE SEMimage of AgNPs biosynthesizedby*L.plantarum*



EIt	W%
C	8.37
Na	5.48
Cl	6.39
Ag	79.77
	100.0

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Figure (6) Point EDS analysis for silver nanoparticle biosynthes

Clinical isolate of *Klebsiella pneumoniae*

A total of (15) clinical isolates of bacteria. were collected from different sites of urinary tract infections and from both gender(male and female) that attended the hospitals in Babylon(Merjan Medical City, Al-Hilla teaching hospital and Public health) during a period from September 2020 to march 2021. All isolates were taken under supervision of subsptilities physician in the hospital.. All samples were streaked on MacConkey's agar and Blood agar, then incubated at 37C° under aerobic conditions for 24 hrs. Culture results were interpreted as being lactose fermenting and non-fermenting bacteria. Lactose fermenting isolates were sub cultured, incubated for an additional overnight. All isolates were examined for colonial morphology, then Gram's staining was used to analyze them microscopically. Biochemical analyses have been used to identify suspected bacterial isolates to the level of species or subspecies (Collee et al. 1996; MacFaddin 2000), and confirmatory detection was done using the Vitek-2 system according to the instructions of the manufacturer.

The Congo Red Method uses a particular kind of formulated solid medium brain heart infusion broth (BHI) combined with 5% sucrose and Congo red for biofilm forming screening. 37 g/l BHI, 50 g/l sucrose, 10 g/l agar No. 1, and 0.8 g/l Congo red stain were used in the medium. Congo red was prepared as a condensed aqueous solution that was autoclaved separately from the other medium constituents at 121 ℃ for 15 minutes. The concentrated solution was applied to cooled agar at 55 °C before autoclaving. At 37 °C, the inoculated plates were incubated aerobically for 24–48 hours. Black colonies with a dry crystalline consistency indicated a promising result. For the most part, weak slime producers remained pink, but the colonies' centers darkened. In the lack of a dry crystalline colonial morphology, the colonies darkened, indicating an indeterminate outcome (Suresh et al., 2016). In BHI agar supplemented with Congo red, the organisms' ability to form biofilms was checked. Exopolysaccharide formation by Klebsiella pneumonia is shown by the presence of black crystalline colonies.

Biofilm Formation by *Klebsiella pneumoniae*:

Biofilm formation by *K. pneumonia* isolates was investigated and the results showed that 10 isolates were strong, 5 isolates had moderate capacity of biofilm formation.

3.1.8: Polymerase chain reaction (PCR) primer sequences

Table (3-8). The size and sequences of primers utilized in current study

Target Gene	sequences of primers (5'- 3')		Product size (bp)	Accession Number	References
mrkD	F	AAGCTATCGCTGTACTTCCGGCA	340	EU682505	Fabrice et al.,2014
	R	GGCGTTGGCGCTCAGATAGG			
Wbbm	F	ATGCGGGTGAGAACAAACCA	120	M55912.1	C.Vuotto et al.,2017
	R	AGCCGCTAACGACATCTGAC			
Wzm	F	TGCCAGTTCGGCCACTAAC	148	AJ430233.1	C.Vuotto et al.,2017
	R	GACAACAATAACCGGGATGG			

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A. adenine, C. cytosine, G. guanine, T. Thymin

Table (3-11) PCR Thermo cycling Conditions of Virulence Factors
PCR Thermocycling condition
Genes in this Study. *mrkD,wbbm,wzm* gene

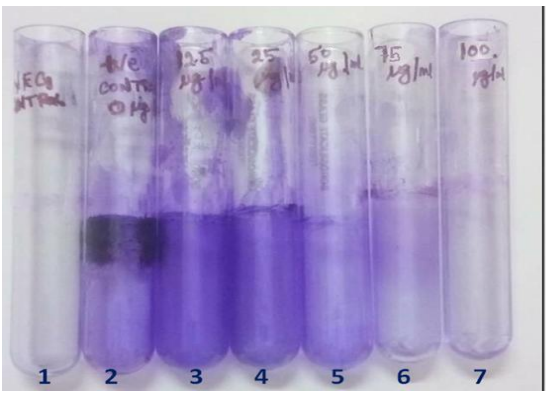
	stage	temperature	Time of incubation	Cycle number
	Initial denaturation	95 C	5 min	1
	denaturation	95 C	30 second	35
	Annealing	58 C	40 second	
	polymerization	72 C	30 second	
	Final polymerization	72 C	5 min	1

Molecular Characterazation of *K. pneumoniae* isolates:
Four selected genes were investigated in this study which play significant role in the pathogenesis andbioflim formation of *Klebsiella pneumonia*. These genes include: *mrk*D, *wzm* and *wbbm*, detection of these gene was established by conventional PCR technique.

Results

The Activity of Antibiofilm of AgNPs through method of the tube:

We analyzed how different concentrations of AgNPs affected biofilm formation in *Klebsiella pneumoniae, E. coli, Salmonella typhi, Enterobacter faecalis, and Pseudomonas aeruginosa*. After 24 hours of treatment, it was discovered that silver nanoparticles inhibit biofilm formation as robust biofilms (+++), resulting in moderate biofilm production at both 12.5 and 25 g / mL AgNPs concentrations, weak (+) production at both 50 and 75 g / mL AgNPs concentrations, and fully prevented the formation of the biofilm at 100 g / mL AgNPs by Escherichia coli, *Klebsiella pneumoniae, Salmonella typhi, and Pseudomonas aeruginosa*.. At doses of 12.5 mcg / mL AgNPs, no biofilm inhibition was observed, indicating a robust biofilm product (+++), but biofilm inhibition was observed at 25 mcg / mL, indicating a mild biofilm product (+++) from a strong biofilm produced (+++). For Salmonella typhi, moderate and weak biofilms were produced at 50 and 75 g/mL AgNPs, respectively, and 100 g/mL AgNPs fully inhibited biofilm formation. The formation of the biofilm was uniformly inhibited for all strains when treated with 100 g/mL AgNPs for up to 24 hours, and stable formation of the biofilm was observed in positive test tubes when treated without AgNPs (0 g/L), also no negative biofilm for all strains. With increasing concentrations of AgNPs, biofilm formation was slowly inhibited (Figure-1) (Table-4.21).



1- Negative control
2- Positive control (0 µg/ml)
3- 12.5 µg/ml
4- 25 µg/ml
5- 50 µg/ml
6- 75 µg/ml
7- 100 µg/ml

Fig (1): The activity of Anti-biofilm of silver nanoparticles with different concentrations by the method of the tube

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Characterization of the Activity of Anti-biofilm of AgNPs over CRA Plates

Several species that contain exogenous polysaccharides have been shown to form biofilms. Microorganisms bind to a surface that is coated in dirt, an exopolysaccharide membrane that defends bacteria from detrimental environmental factors. *Escherichia coli,Klebsiella pneumoniae, Salmonella typhi, Pseudomonas aeruginosa*, and fecal intestinal strains were grown in Brain Heart Infusion Agar to see whether they could form biofilms. After 24 hours of incubation, additive Congo Red (BHIC) with and without silver nanoparticles are tested. Colonies grown in the absence of AgNPs in the medium appeared as dry, crystalline black colonies, signaling the synthesis of exopolysaccharides, a prerequisite for biofilm creation.

When the species in BHIC were grown with AgNPs, the findings were different. The organisms tended to evolve in the existence of low concentrations of AgNPs (12.5 g/mL), but AgNP treatment prevented glycocalyx matrix synthesis, suggesting that dry black crystal colonies were not present. At high concentrations of AgNPs, bacterial growth was observed to be inhibited by more than 90%. The organism is unable to form a biofilm while the replication of the glycocalyx matrix is halted. Also, it was discovered that 25 g/ml AgNPs greatly slowed biofilm formation without influencing viability, while 100 g/ml AgNPs stopped biofilm formation entirely and inhibited organism development (Figure-2) Biofilm formation was slightly delayed without impacting viability, while biofilm formation was totally stopped and the organism's growth was inhibited at 100 g/ml [Figure-2],(Table-1)

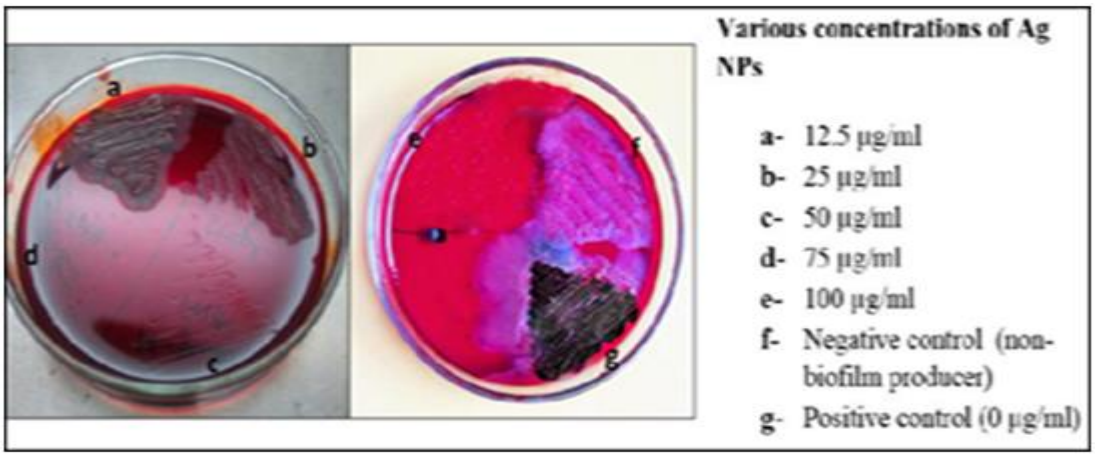

Fig 2: The activity of anti-biofilm of silver nanoparticles at various concentrations by the Congo red agar method.

					Tube method				Congo red agar method				
S. No	**SBP-MDR strains**	**Negative control**	**Positive control (0 µg/ml)**	**Different concentration of biosynthesized AgNPs in µg/ml**									
				12.5	**25**	**50**	**75**	**100**	**12.5**	**25**	**50**	**75**	**100**
		Robust multidrug-resistant strains produced on biofilms											
	Salmonella typhi	–	+++	++	++	+	+	--	**B**	**B**	**AB**	**R**	--
	P.aeruginosa	–	+++	++	++	+	+	--	**B**	**B**	**AB**	**R**	--
	K. pneumoniae	–	+++	++	++	+	+	--	**B**	**B**	**AB**	**R**	--
	E. coli	–	+++	++	++	+	+	--	**B**	**B**	**AB**	**R**	--
	Enterobacter faecalis	–	+++	+++	++	++	+	--	**VB**	**B**	**B**	**R**	--

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MDR - multidrug-resistant strains; Mg/ml - micrograms per milliliter; AgNPs - silver nanoparticles; SBP - strong biofilm production; MDR - multidrug-resistant strains; Mg/ml - micrograms per milliliter; Mg/ml - micrograms per milliliter; Mg / m VB stands for very black colonies, while B stands for black colonies. R-red colonies; AB-almost black colonies. (+++/VB) indicates a solid biofilm product; (++/B) indicates a moderate biofilm product; (+/AB) indicates a weak biofilm product; (-/p) indicates a non-biofilm product.

Detection of *mrk*D

MrkD gene (Mannose resistant Klebsiella polypeptide A) type 3 fimbriae-dependent adherence could be a major virulence determinant in nosocomial infections, as it could be the first step in *Klebsiella pneumoniae* colonization of non-biological surfaces and the formation of the biofilm. However, the mrk D gene has been found in some extremely adherent strains (Nathalie et al; 2003). The type 3 fimbria main subunit is a major contributor to the biofilm of *K. pneumoniae*. mrkD (Regulator of mucoid phenotype A) has been related to the hyper muscoviscosity phenotype in K. pneumoniae, which is responsible for the bacterial capsule, which has been shown to be more normal in liver abscess strains than in bacteremia isolates (Yu et al., 2006).The findings showed that the mrkD gene was found in 20 positive isolates (100 percent). It is a key factor in the virulence of K. pneumoniae strains, and it is regulated by plasmids that promote a strongly mucoviscous phenotype and the capsular polysaccharide synthesis regulator (Rivero et al.,2010).

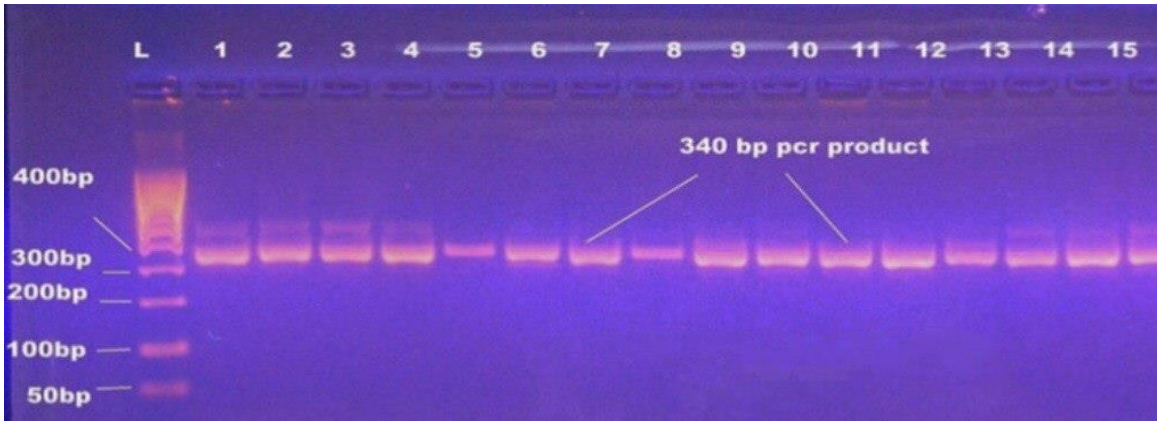

Figure (4-4): Agarose gel electrophoresis of 15 bacterial spesimens of mrkD gene amplicon product in *Klebsiella pneumoniae* lane 1-15refer to isolate`s number. (The size of ladder is 100pb (Bioneer), the volte of electrophoresis is 80V for 60minuts, the size of agarose is 1.5%, the size of bands is 340bp).

Detection of wbbm

In this study, the wbbm gene was discovered in (100 percent) wbbm mutant strains of many gram-negative bacteria. Lipopolysaccharide (LPS) is a main structural and immunodominant molecule in gram-negative bacteria's outer membrane. The three main regions of LPS are lipid A, core oligosaccharide, and O antigen. The O antigen, which is made up of polymer oligosaccharide repeating units, is the most external part of LPS.The high chemical diversity of O antigens is mirrored in genetic variation in the genes included in O-antigen biosynthesis, known as the wb cluster. The biology of O-antigen biosynthesis in Enterobacteriaceae has been thoroughly investigated, and it has been discovered that wb clusters ordinarily include genes included in activated sugar biosynthesis, glycosyltransferases, O-antigen polymerases, and O-antigen export (Kelly et al., 2019).

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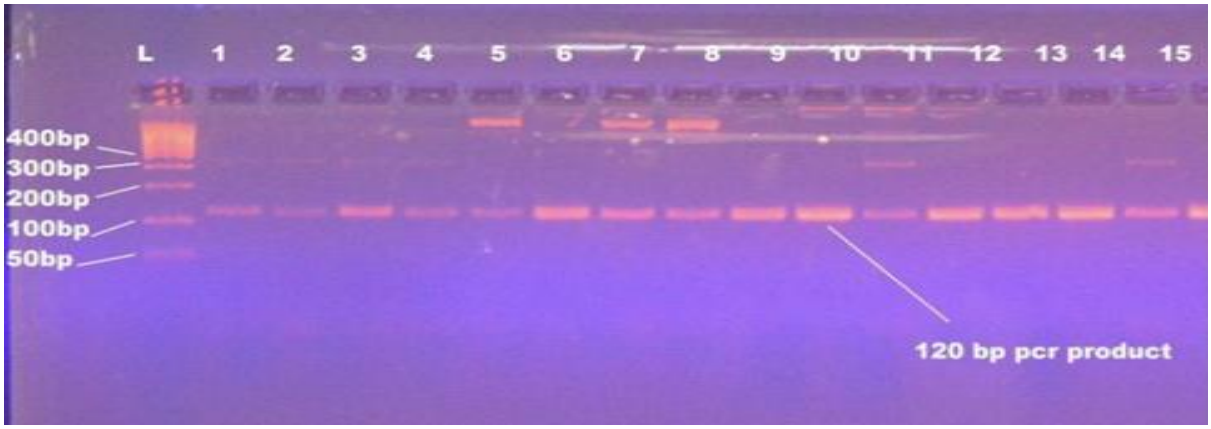

Figure (4-6): Agarose gel electrophoresis of bacterial specimens of the genomic amplicon product wbbm in Klebsiella pneumoniae lane 1-15 indicates the isolation number. (Scale of scale 100pb, volte of electrophoresis 80V for 60 min, the volume of agarose 1.5%, size of bands 120 bps).

Detection of wzm

The current study explained that the increase serum survival wzm gene was observed in (100%) isolate of all *Klebsiella pneumonia* isolates figure (4.6) This result dis agree with (Al-Janabi *et al* 2018) who found 26 isolates the three to five positive of *K. pneumoniae* (60%) isolates. The increased serum survival protein (wzm *gene*) has an action in defense to serum complemen .

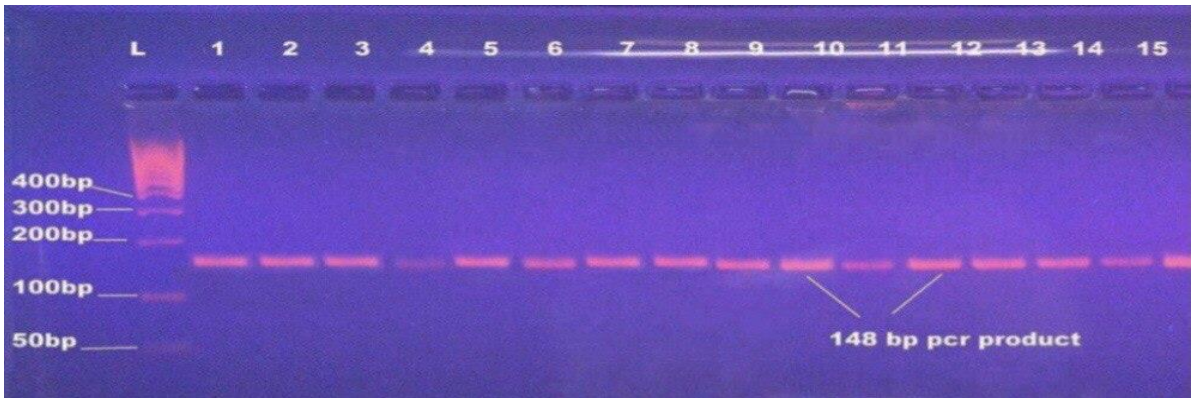

Figure (4-6): Agarose gel electrophoresis of bacterial specimens of the WZM gene amplicon product in Klebsiella pneumoniae 1-15 indicates the isolation of the number. (Ladder volume 100pb, 80V electrophoresis volt for 60minuts, 1.5% agarose volume, and bands volume 148bp).

Correlation between Phenotype and Genotype:

The resort to be abundant in resistance to serum influence Olling. (1977). The results correlation between phenotype test of capsules, biofilm, serum resistance and ESBL with the genotype test of (*mrkD, wbbm*, and *wzm*) shown in the table (4.6). The result showed strong correlation between phenotype and genotype for biofilm and serum resistance capsule, and ESBL respectively. Usual serum has bactericidal action in contradiction of expanse of gram-negative bacteria (Seigfried *et al* 1994). The pathogenicity of bacteria is partially a function of their capability to avoid the bactericidal influence of serum, which is arbitrated by the cascade of the complement. Endosymbiotic bacteria are usually susceptible to the bactericidal influence of serum, while hospitalized bacteria of current work were partially matching with the results of similar studies, which performed in Iraq (Al- Janabi, *et al* 2018).
Biofilms are bacterial populations of adhesive organisms to each other on a target surface.

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Biofilm production shields bacteria from hydrodynamic stream circumstances, for instance in the urinary tract, and in contradiction of phagocytosis and patient resistance devices, add to antibiotics (Hanna *et al*., 2003). Greater than 50% of bacterial infections, which involved biofilm production (Costerton *et al*., 1999**)** A progression of numerous accurately, strongly controlled actions are necessary for accurate biofilm production.
The prevalence of *wzm* among bacteria was the second stage after biofilm and this result showed the importance of *wzm* gene in the pathogenicity of isolated bacteria. Usual serum has bactericidal action in contradiction of expanse of gram- negative bacteria (Seigfried *et al* 1994). The pathogenicity of bacteria is partially a function of their capability to avoid the bactericidal influence of serum, which is arbitrated by the cascade of the complement.

Discussion

Antimicrobials are becoming more immune to a wide range of chemical antimicrobial agents. As a result, a new approach for countering drug resistance in different microorganisms is critical. Due to their ability to inhibit microorganism growth, silver ions, and silver salts that utilized as antimicrobial agents in a number of fields for decades (Silver et al.,1996). However, there are several risks of utilizing Ag ions or Ag salts as antimicrobial agents. One of the most possible reasons is the intervening effects of salts. This kind of restriction could be removed using silver in nano form. Biofilm inhibition declined in this analysis as the concentration of AgNPs in the sample increased. When the tubes were treated with AgNPs at concentrations of 12.5, 25, 50, and 75 g/ml, biofilm was formed [Figure-6], but none of the strains at 100 g/ml containing tubes formed biofilm [Figure-6].When Asaduzzaman et al. investigated the influence of AgNPs at different concentrations on the production of biofilm by K. pneumoniae, they observed similar results. At doses of 5, 10, and 20 g/ml of AgNPs, K. pneumoniae shaped biofilm, but no biofilm was detected at 40 and 80 g/ml of AgNPs. In the test tube that did not contain AgNPs, however, biofilm was detected. (Asaduzzaman et al .,2016)
However, Growing the organism on CRA supplemented with and without AgNPs was utilized to test the anti-biofilm efficacy of AgNPs. The organisms appeared as dry crystalline black colonies when grown without AgNPs, suggesting the production of exopolysaccharides (EPS), which is needed for the creation of biofilm Figure-2(g)]. The organisms that were cultivated with AgNPs, on the other hand, did not survive. The organisms continued to grow through treatment with lower concentrations of AgNPs (12.5 g/ml), but the AgNPs treatment prevented the synthesis of glycocalyx matrix, as shown by the lack of dry crystalline black colonies. At higher AgNP concentrations (100 g/ml), however, almost no growth was observed [Figure-2(c-e)]. As a result, when exopolysaccharide synthesis is halted, the organism is unable to form biofilm. Kalishwaralal et al. published similar findings against Pseudomonas aeruginosa and Staphylococcus epidermidis biofilms, finding that 100 nM of AgNPs decreased biofilms by 95-98 percent (Kalishwaralal et al .,2010). It has also been stated in the literature that providing an AgNPs coating on the surface of medical devices helps to prevent adhesion of the bacterial and eventual formation of the biofilm over the devices (Knetsch et al .,2011).
The findings are in agreement with the previous study findings on nanocrystalline silver's anti-biofilm behavior done by Kostenko et al. Nanocrystalline silver significantly reduced the number of viable cells in the biofilms analyzed.

Only a few techniques are available for detecting biofilms on medical equipment or structures. Staining both the bacteria and the glycocalyx is impossible, making bacterial biofilm demonstration difficult. According to Kostenko et al., Acticoat nanocrystalline silver has the best anti-biofilm efficacy as compared to Aquacel silver and Silverlon, and silver concentration alone cannot account for silver dressings' anti-biofilm efficacy (Kostenko et al .,2010).
Ansari et al. found that when exopolysaccharide synthesis is blocked, E. coli and K. pneumonia biofilms cannot shape on CRA medium and also in SEM observation by

modifying the morphology of biofilms due to the roughness of the cell surface being damaged by 20 g/ml AgNPs. According to Ansari et al., the existance of water channels in the biofilm may explain the inhibitory influence of AgNPs on the current biofilm. AgNPs can diffuse directly through the exopolysaccharides layer through the pores and impart antimicrobial activity because all biofilms have water channels (pores) for nutrient transportation (Ansari et al .,2013).
The relative surface area of a silver particle is increased when it is reduced to the nanoscale level, resulting in higher Ag+ release rates than for elemental silver particles (Dunn et al .,2004).
Furthermore, nanoparticles have a greater ability to adhere to and infiltrate membranes of the bacterial, accumulating within cells and releasing silver ions continuously (Ansari et al.,2013), (Rai et al2009). (Ansari et al .,2014)

Conclusion

When assuming infections that take on the biofilm phenotype are notoriously difficult to treat. Antibiotic therapy may be ineffective against biofilm infections, or they may respond initially only to relapse weeks or months later. In those circumstances, invasive procedures like surgical removal and reconstruction of the contaminated tissue or system might be needed. As a result, screening for biofilm development is needed for the appropriate treatment of biofilm infections that cause clinical isolates. Just a few techniques can be used to detect the existence of biofilms over medical devices or surfaces. It's difficult to stain both the bacteria and the glycocalyx, which makes demonstrating bacterial biofilms difficult.

The nature of clinical specimens is associated with biofilm formation. Silver nanoparticles with small particles have higher anti-biofilm activity, which is dependent on concentration. In the current study, Silver nanoparticles have a high anti-biofilm function. Silver nanoparticles have a different anti-biofilm effect on different bacterial isolates, and K. pneumonia is more susceptible to nanoparticles. Silver nanoparticles may be utilized to prevent bacterial biofilms from forming, which can be useful in the treatment of infectious diseases caused by biofilms. We recommend that most research be done on this issue, especially in vivo and clinical trials to determine toxicity levels before using silver nanoparticles in the treatment of infections of the biofilm.

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