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Different Methods for Detection Sliver Nanoparticles Produced by *Proteus mirabilis* Bacteria

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Abstract : In this study, eighty mid-stream urine samples were collected from patients suffering from catheter associated urinary tract Infections (CAUTI) who visit urological consultant clinic of Hillah Teaching Hospital in Hillah, Babylon province- Iraq during a period from August2014 and January 2015. All urine samples were subjected for standard bacteriological procedures to diagnostic bacteria. The results show that 30/80 (37.5%) give positive culture for *Proteus mirabilis.*

Detection the ability of this bacteria to the reduction of Ag+ to elemental silver nanoparticles Ag^0 was characterized by X-ray diffraction (XRD), UV-Vis spectrum, Fourier Transform Infrared Spectroscopy and Scanning Electron Microscopy(SEM).The molecular detection by using PCR primer ,the primer pairs used in this study first time using Work bench-primer3software.

Keywords : AgNPs, XRD, SEM, FTIR , *Proteus .mirabilis.*

Introduction:

Catheter-associated urinary tract infection (CAUTI) is the most common nosocomial infection, accounting for more than 1 million cases each year in the US hospitals and nursing homes. The significant number of infections and dissemination of resistant bacteria in hospitals make it important to find ways to decrease their incidence[1]

The *Proteus* genus goes to the Proteeae tribe in the family of Enterobacteriaceae, this tribe contains of three types: *Proteus, Providencia*and *Morganella*. These bacteria are gram negative rod measuring (0.4–0.8) μm in diameter and (1–3) μm in length, motile by peritrichous flagella, non-spore forming ,non-capsulated, facultative anaerobic, major isolates have fimbriae, The genus of *Proteus* contains of four *spp*: *P.mirabilis*, *P. vulgaris*, *P.myxofaciens*.and *P. penneri*[2].

The nanotechnology field is an considerably evolving field as a consequence of its diverse ranging uses in many parts of technology and science[3,4]. The meaning of nanotechnology is the formation, manipulation and synthesis of materials at a measure lesser than1 μm .The word "nano" is derivative from a Greek word meaning dwarf or extremely small[5].

Biological synthesis of various metal nanoparticles by using prokaryotic as well as eukaryotic organisms including bacteria, fungi and plants[6].

However, among various organisms studied until to date, prokaryotic the choice of organism for biological synthesis of nanoparticles. This is predominantly because prokaryotes offer well-defined advantages over eukaryotic organisms such as easy handling, ease of downstream processing and ease of genetic manipulation[7].

Bacterial synthesis of silver nanoparticles (AgNPs) is particularly attractive from microbiology perspective due to existence of well-known silver resistance machinery in few silver resistant bacterial species, thus making their study significantly important for biomedical applications[8].

More recently, silver nanoparticle have been shown to locally increase light by 10–100 times ,leading to surface-enhanced Raman scattering (SERS), with improvement factors on the order of 10^6–10^8 .[9] Silver nanoparticles have been successfully synthesized from gram negative bacteria like *E.coli*. Silver nanoparticles have a characteristic feature of absorbing light at a wavelength of 420 nm[8].

The biological devices used for the synthesis of nanoparticles contain together extracellular and intracellular methods[10] .

Moreover, silver nanoparticles have remained an attractive choice of nanomaterial because of their ability of encompassing broad application area from electronics to medicine to food technology[11].

Biological agents used for the creation of nanoparticles include mostly microorganisms and plants[9].

The occurrence of the nitrate reductase enzyme is the greatest widely accepted mechanism of AgNPs. The enzyme transforms nitrate into nitrite. Nitrate is transformed into nitrite through the reduction and the electron is transferred to the Ag^+ ion; hence, the Ag^+ ion is reduced to Silvernanoparticles (Ag^0). This has been said to be observed in Bacillus licheniformis which is known to secrete NADPH and NADPH-dependent enzymes like nitrate reductase that successfully converts Ag+ to Ag^0,[12].

Biological way for the synthesis of nanoprticles is also known as “green synthesis”. Green synthesis is preferred over the rest because chemical method needs both weak and strong reducing agents and protective agents which are toxic to environment, but biological method is nontoxic and is good in regard to the environment. It has been also found to be of low production rate in chemical method[12].

Materials and Methods:

1.Patient and Samples

Eighty mid-stream urine samples were collected from patients suffering from Catheter Associated Urinary Tract Infections (CAUTI) who visit urological consultant clinic of Hillah Teaching Hospital in Hillah,Babylon province- Iraq, biochemical diagnostic standard test, all samples were subjected to standard bacteriological procedure including culturing onblood and MacConkey's agar plates for isolation and incubated for 24-48 hours at 37 ^{O}C[13]. All suspected Gram negative isolates were screening by traditionally tests and then confirmed By Viteck 2 compact system (Biomérieux).

2.DNA Extraction, Primer Designing and PCR Conditions

All *Proteus mirabilis* isolates were subjected for DNA extraction according to the protocol provided by manufacturer (Geneaid/Taiwan). The primer pair used to investigate silE gene was designed in this study using Workbench - Primer 3 software. The primer sequence

F: ATATCCATGAGCGGGTCAAC R:CAACTGCAGCTCTTTCATGC. The PCR product size was 280 bp.The protocol type: was Simple 3-step PCR protocol illustrated in table(1).

Table (1):The cycling conditions of phylogeny groups

Steps	Temperature	Time	No. of cycles
Initial denaturation	95 ^{o}C	2 min	1
Denaturation	95 ^{o}C	30 sec	30
Annealing	59 ^{o}C	30 sec	
Extension	72 ^{o}C	30 sec	
Final extension	72 ^{o}C	5 min	1
Hold	4 ^{o}C	10	

Table (2): The 30 µl PCR mix

Item	Volume
Master mix	15 µl
Target DNA	5 µl
Forward Primer (10pm/ µl)	2.5 µl
Reverse Primer (10pm/ µl)	2.5 µl
Nuclease free water	5 µl
Total volume	30 µl

3. Methods of Biosynthesis and Detection of Silver Nanoparticles

The method of silver nanoparticles production by *Proteus mirabilis*was achieved as indicated by[14]. As follow:the *Proteus mirabilis*isolates were initially grown at 37 C^0 for 24 h in a 500-mL Erlenmeyer flask that contained LB broth (100 mL) in a shaker incubator set at 200 rpm and then The bacterial growth were incubated withaqueous 5 mM solutions of AgNO3 at 37C in a shaker incubator at 200 rpm in the dark, and the reactions were carried outfor up to 120 h (5 days).The extracellular synthesis of AgNPs was initially detected by visual inspection of the culture flask for a change in color of culture medium from clear light-yellow to brown/green. The separations of AgNPs from bacterial cells were performed by centrifugation of aliquots of culture supernatants (1.5 mL) at 3000 rpm for 6 min at 25C.

The UV-vis analysis was done as follow, the AgNPs suspensions were diluted 10 times using deionized water at every time point and UV-vis spectra were obtained. For X-RayDiffraction(XRD)andFourier transform-infrared spectroscopy(FTIR) analysis according to[16], the samples wereprepared by precipitating AgNPs obtained after of biosynthesis at 13,000 rpm for 20 min, followed by four washings with deionized water, and drop casting the samples onto a glass substrate.

For SEM analysis, AgNPs samples obtained after of reaction were prepared by drop casting the colloidal suspensions of AgNPs onto carbon-coated Cu grids followed by drying under air for 24 hours[15].

Results and Disccution:

The urine samples were mainly grown onto blood and MacConkey's agar plates for isolation and incubated for 24-48 hr at 37°C[14]. All samples culturing on traditional and conventional media. A total of 59(73.7%) positive cultures, just thirty 30 (37.5 %) showed positive for *Proteus mirabilis* Figure(1). The identification of these isolates depends on the morphological properties such as colony size, shape, color, natural of pigments, transparency, edge, elevation, texture and lactose fermentation on MacConkey agar on blood agar. The isolates are pale non-lactose fermented with fishy odor, motile(swarming phenomenon) and cause β-hemolysis colonies on blood agar, according to[16].

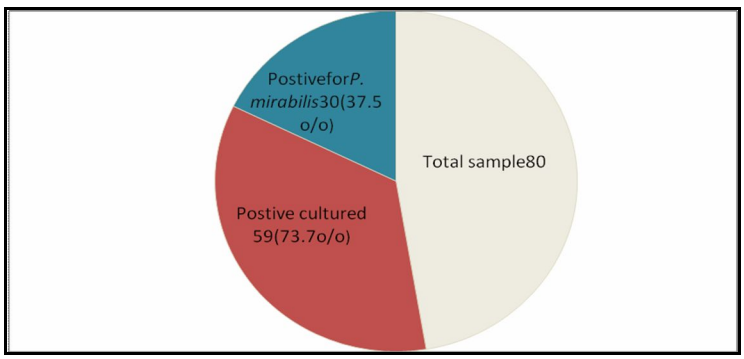


Figure (1) :Percentage of *Proteus mirabilis*in patients

Wholly the 30 isolates of *Proteus mirabilis* displayed positive marks to the biochemical tests, phenylalanine deaminase, catalase, hydrogen sulfide H2S,gas production ,hemolysin ,methyl red ,gelatin, urease

and KIA, but all were oxidase, citrate utilization test ,indole productionVogesProskauer are negative.These isolates were motile they appear swarming phenomenon on agar . Moreover *Proteus* isolates were incapable to ferment lactose, mannitol and maltose but ferment glucose only as illustrated in table (3).

Table (3): Biochemical test of *Proteus mirabilis*

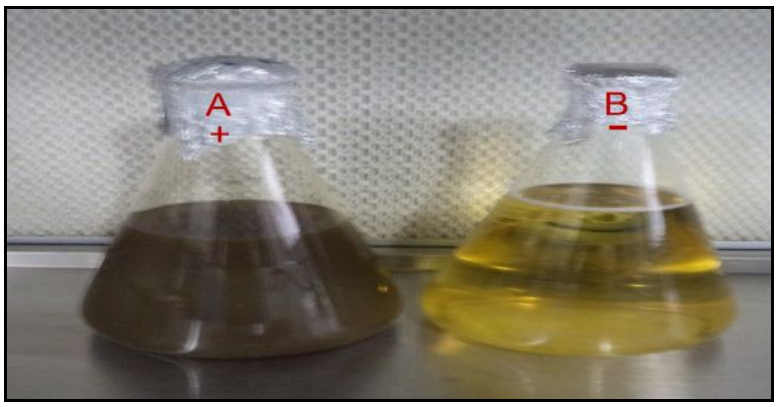
No	tests		Results
1	Catalase		+
2	Oxidase		-
3	Kliglers Iron Agar (KIA)	Hydrogen sulfide H2S	+
		Slope	Alkaline
		Bottom	Acid
		Gas production	+
4	Urease production		+
5	Hemolysin production		+
6	Indole production		-
7	Methyl red		+
8	Voges –Proskauer		-
9	Citrate (Simmons)		-
10	Gelatin		+
11	Maltose fermentation		-
12	Glucose fermentation		+
13	Lactose fermentation		-
14	Mannitol fermentation		-
15	Phenylalanine deminase		+

Also, the system Vitektwo was used to confirm the results of identification. There was a difference between species but the result probability between (95-99%) are show, in table (4).

Table (4) :Result of Vitek tow system for *P.mirabilis*.

No. of well	Symbol of test	Result	No. of well	Symbol of test	Result
2	APP A	-	**33**	SAC	-
3	ADO	-	**34**	dTAG	-
4	PyrA	-	**35**	dTRE	+
5	IARL	-	**36**	CIT	+
7	dCEL	-	**37**	MNT	-
9	BGAL	-	**39**	5KG	-
10	H_2S	+	**40**	ILATK	-
11	BNAG	-	**41**	AGLU	-
12	AGLTP	-	**42**	SUCT	+
13	dGLU	+	**43**	NAGA	-
14	GGT	+	**44**	AGAL	-
15	OFF	+	**45**	PHOS	+
17	BGLU	-	**46**	GlyA	-
18	dMAL	-	**47**	ODC	+
19	dMAN	-	**48**	LDC	-
20	dMNE	-	**53**	IHISa	-
21	BXYL	-	**56**	CMT	+
22	BAlap	-	**57**	BGUR	-
23	ProA	-	**58**	O129R	+
26	LIP	-	**59**	GGAA	-
27	PLE	-	**61**	IMLTa	-
29	TyrA	+	**62**	ELLM	-
31	URE	+	**64**	ILATa	-
32	**dSOR**	-			

Allisolate of *Proteusmirabilis* were capable to synthesize extracellular Ag nanoparticles. To assume this study *Proteus* isolates were exposed to 5 mM colorless $AgNO_3$ solutions ,*Proteus mirabilis* formed dark brown colored solutions within 20 h of reaction figure(2), the color of the solutions did not significantly change from that point forward (except in intensity), even after persistent the reaction for up to 5 days**.**



Figure(2): Qualitative test (coloring test)A: medium with AgNO3 (5mM) positive result: brownish color B: control medium withoutAgNO3 (5mM) negative result: yellow color

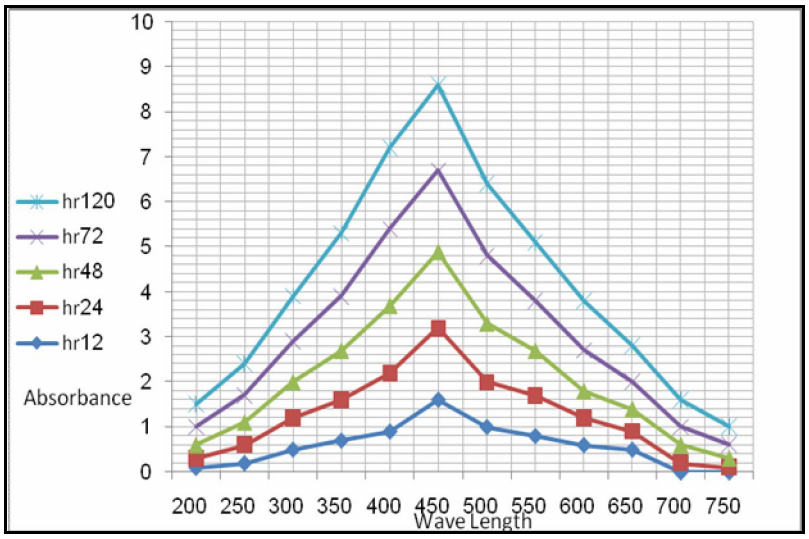


Figure (3) :UV-VIS absorbance spectroscopy for AgNPs from *Proteus mirabilis*

Figure (3) shows the UV-vis absorbance spectra of colloidal solutions acquired after reaction of all *Proteus* isolates with 5 mM $AgNO_3$ for zero, 12, 24 ,72and 120hr. The occurrence of a characteristic Ag Surface Plasmon Resonance (SPR) between 400 and 500 nm is clearly marked in all the samples, thus confirming the creation of extracellular AgNPs by all *Proteus mirabilis*[6]. The crystallography of AgNPs made by several isolates of *Proteus* after of reaction was examined by XRD. As is evident from XRD patterns in Figures (4 and 5), extracellular AgNPs synthesized by *Proteus mirabilis* are greatly crystalline in nature, that could be perfectly indexed to the (111), (200), (220) and (311)in peaks at 2Theta (37), (44), (64), (77),crystalline silver so this result similarity with[17].

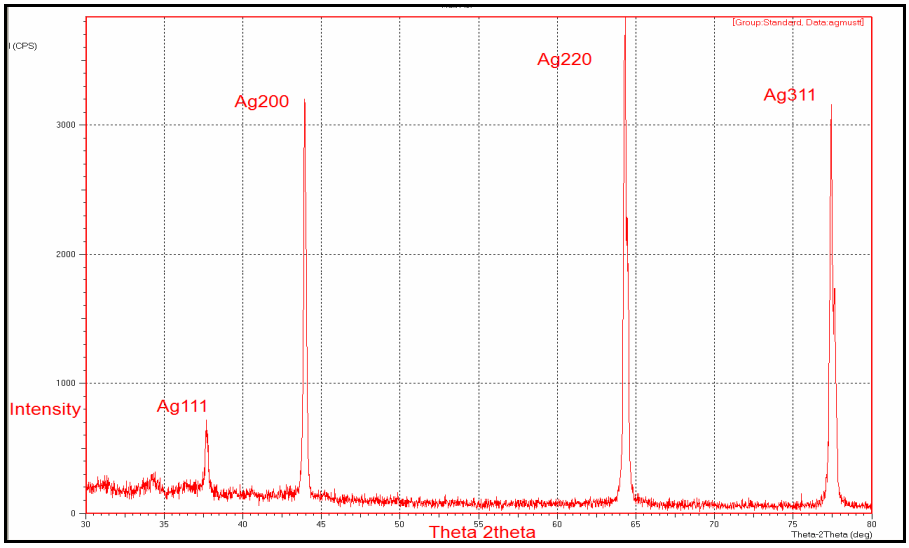


Figure (4) :X-ray diffraction results for *Proteus mirabilis*, XRD patterns recorded showing 4 sharp peaks corresponding to the diffraction from111,200,220and311planesof sil

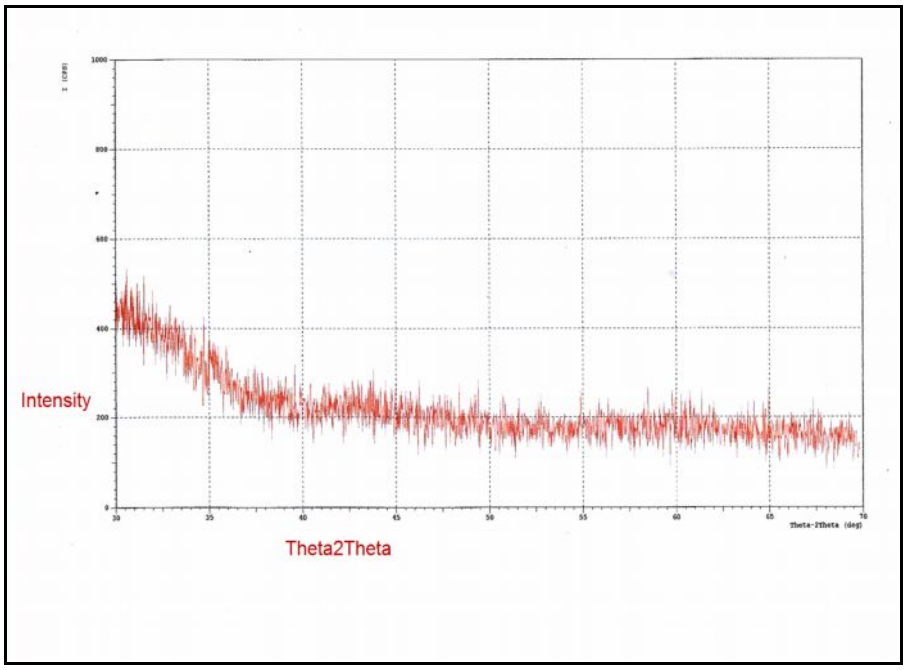


Figure (5) :X-ray diffraction results for *Proteus mirabilis* without AgNO3 (control)

FTIR measurements were carried out to identify possible interaction between sliver nanoparticles and protein molecules, which could account for AgNPs formed by *Proteus mirabilis* figure(6) .The linkage of amides among amino acid residues in protein provide increase to the recognized signatures in the infrared region of electromagnetic spectrum.

The bands detected at 341400cm^{-1},assined to hydroxyl (OH)group. the slight bands detected at 2960.73 cm^{-1} and 2927.94 cm^{-1} symbolize the (CH2)groups ,the bands observed at 1651.07cm^{-1},refered to carbonyl groups(C=O),the bands detected at 1070.49cm^{-1},assigned to(CO)groups[18-22].

The overall observation approves the presence of protein in sample of AgNPs. It has also been reported later that protein can bind to nanoparticles it her concluded their free cysteine residues or amine groups, this result similarity with[17]. who detected sliver nanoparticles produced by extracellular biosynthesis of AgNPs by the Bacteriu*m Proteus mirabilis by* Fourier Transform Infrared .

Scanning Electron Microscopy (SEM) analysis image figure(7) provided further understanding into the form and size of the nanoparticles[23,24]. It is apparent from the figure that the biosynthesized silver nanoparticles are in small and spherical in shape. Silver nanoparticles in the range of 20–35nm by *Proteus mirabilis.* This result agree with reference[15]. result who found that SEM images that silver nanoparticles by an autochthonous strain of *Proteus* were pseudo-spherical in shape, and have different size according to different bio groups.

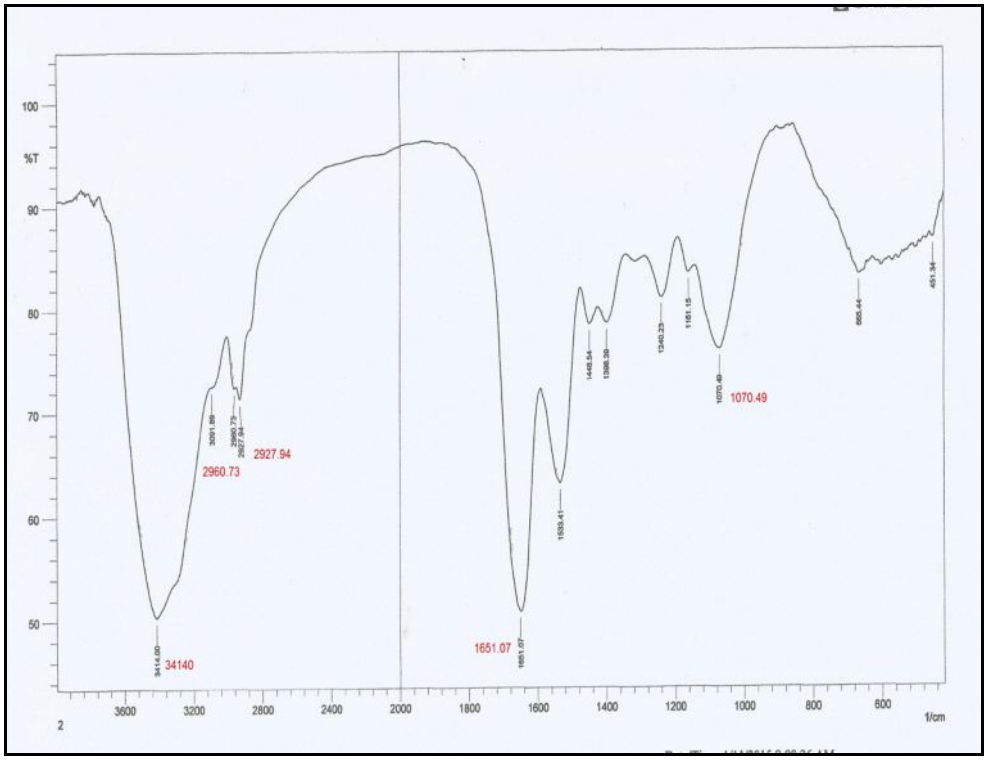


Figure (6): FTIR spectra pattern of dried powder AgNPs synthesized by the reaction of 5 mM aqueous AgNO3 solution with *P. mirabilis*

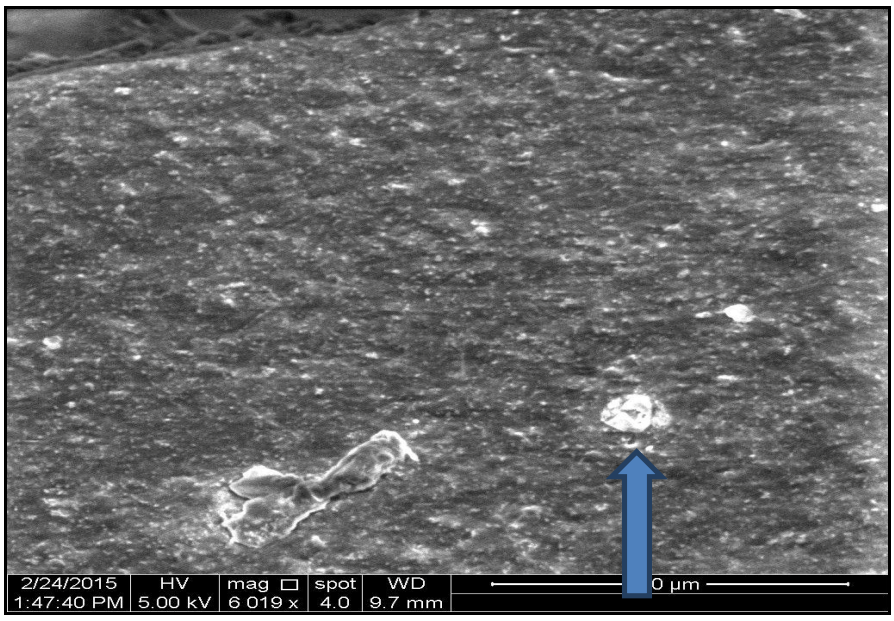


Figure (7) :Scanning electron microscopy (SEM) image of extracellular AgNPs formed by *Proteus mirabilis*.

Silver composites are used as antimicrobial agents in medicine and bacteria that progress resistance to silver cations (Ag^{+}) attitude problems like to those of antibiotic-resistant bacteria, these resistance to silver Ag^{+} apply by silver resistance genes (*sil*E).

Silver nanoparticles were detected in most isolates of *Proteus mirabilis*. The molecular detection by using PCR primers The primer pairs used in this study were designed for first time using Workbench primer 3 software. Formation of AgNPs by *Proteus mirabilis* refer to resistance of these bacteria to incoming silver ion a(Ag^{+}) and transform to metallic AgNPs this confirm presence silver resistance gene (*sil*E) in *Proteus mirabilis* bacteria .This result support with result obtained by (Al-Harbi*etal*.,2014)who investigated presence silver resistance gene(silE) in *Proteus mirabilis* through ability these bacteria to synthesis AgNPs, and decide with[6,14] who investigated presence silver resistance gene (silE) in *Morganellamorganii*as shown in figure(7).

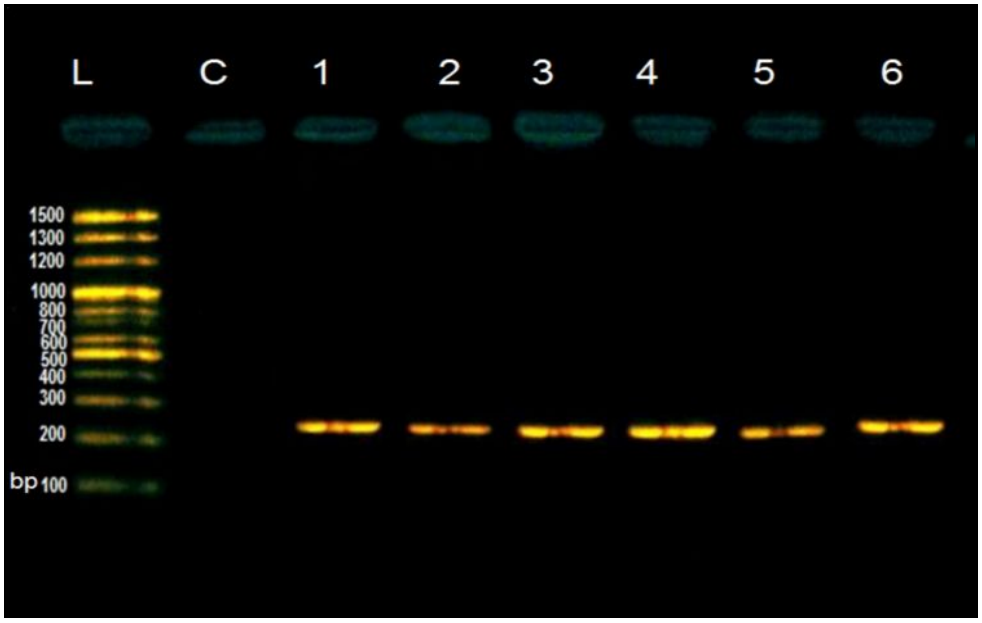


Figure (7): Gel Electrophoresis of the amplified products of *P.mirabilis* (*silE*) gene on(1%) agarose for 1hr to70 voltageL:Ladder100bp C:control represents negative control for *silE* gene1,2, 3, 4, 5, 6, represent sample no. of positive results for *silE* gene.

Conclusion

1. The ability of *Proteus mirabilis* to biosynthesis of sliver nanoparticles by the reduction of silver ion to silver nanoparticles (Ag^{+} to Ag^{0}).
2. Silver nanoparticles features detection by XRD, FTIR, UV-Vis and SEM.
3. Detection the (siLE)gene responsiple of silver nanoparticles synthesis in *Proteus mirabilis*.

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