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Synthesis, Antibacterial activity, and Genotoxicity of Silver Nanoparticles

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ABSTRACT

The aim of this work was to evaluate the antibacterial activity of two types of silver nanoparticles which were prepared within a network of two hydrogels composed of Poly Vinyl Pyrrolidone PVP/Acrylamide/Acetic acid/Glycerol (Silver Nanoparticles 1 AgNP 1) and PVP/Acrylamide/Acetic acid (Silver Nanoparticles 2 AgNP2) via gamma radiation dose at 25 kGy. Furthermore, High Resolution Transmission electron microscopy (HRTEM), was used to investigate the prepared AgNPs with average size around 17.5 and 22.5 nm for AgNP1 and AgNP2 respectively. Silver Nanoparticles, antibacterial and genotoxic effects were examined on *Escherichia coli* ATCC 8739, *Salmonella enterica* ATCC 14028 and *Shigella sonnei* ATCC 29930 as examples for Gram negative bacteria and one Gram-positive bacteria *Staphylococcus aureus* ATCC 6538 as tested organisms. Silver nanoparticles (AgNP1 and AgNP2) showed strong antibacterial activity against the growth of the four bacteria tested. Gram-negative bacteria were more affected than Gram-positive bacteria. and the most sensitive was *Shigella sonnei* and AgNP1 was more effective than AgNP2. Effect of AgNP1 and AgNP2 on the DNA of the four bacteria was examined using 11 ISSR primers which produced 35 and 29 polymorphic bands (disappeared and new bands appeared) in *E. coli*, 18 and 17 polymorphic bands in *Salmonella enterica* and 34 and 32 in *Shigella sonnei*, respectively. In contrast, Gram-positive bacteria *Staphylococcus aureus* displayed the largest polymorphic bands numbers (38 and 49 bands) compared to Gram-negative. Additionally, all samples treated with AgNP1 or AgNP2 showed a decrease in Band Sharing Index (BSI) and Genetic Template Stability (GTS) according to ISSR profile analysis.

1. Introduction

According to [25], nanomaterials are receiving increased attention in the scientific community for applications in biomedical devices, medicine, cosmetics, renewable energy, and environmental remediation. Silver revealed a strong toxicity against a large variety of microbes [5, 7, 8]. Shape, charge, surface, and size of AgNPs are the main factors that influence their antimicrobial properties. Generally, particles less than 100 nm are referred to as nanoparticles that provide them with unique properties [11, 15]. AgNPs have the ability to inhibit the growth of bacteria, including *Proteus vulgaris, Escherichia coli, Staphylococcus aureus,* and *Streptococcus mutans* according to [12, 13, 14].

Because peptidoglycan, a crucial component of Gram-positive bacteria's membranes, differs structurally from Gram-negative bacteria, the effect of nanoparticles against Gram-negative bacteria was more pronounced. [26].To find the right concentrations for nanoparticles and learn more about their genotoxicity as safe disinfectants and alternatives to antibiotics, more research on nanoparticles is needed. Silver nanoparticles can be used to produce a new class of antimicrobials which opens a new way to fight pathogenic bacteria. It can be applied to crops as fertilizers or bactericides [12, 19]. However, to certify food safety, potential health effects must be considered [31].

In this study, silver nanoparticles synthesis and characterization that embedded in two different copolymer compositions of polyvinyl pyrrolidone (PVP)/acrylamide hydrogels using simultaneous gamma irradiation technique were carried out. The cytotoxic and genotoxic silver nanoparticles effect on four bacterial strains (three Gram-negative and one Gram-positive) were evaluated using ISSR- PCR technique. For a number of reasons, ISSR (Inter Simple Sequence Repeat) is one of the most widely used DNA fingerprinting methods. ISSR markers have proven useful in a variety of fields. ISSR based markers have utility in the fields like genetics, taxonomy, physiology, embryology etc.

2.1. Materials and Methods

2.1. Preparation of polyvinyl pyrrolidine (PVP)/Acrylamide hydrogels containing silver nanoparticles

Two different copolymer compositions of PVP/acrylamide hydrogels containing silver nanoparticles were synthesized and prepared using ionizing radiation.

The first type of silver nanoparticles (AgNP1) was composed of PVP/Acrylamide/Acetic acid/Glycerol/Silver nanoparticles, while the second type (AgNP2) was prepared by the same procedures without adding glycerol (AgNP2) In 138 ml of distilled water, a mixture of 15% PVP (from Universal Fine Chemicals) and 25% acrylamide (from Alpha Chemika) was prepared in the ratio (40:60), then a mixture of 5% acetic acid (from Loba Chemie) and 5% glycerol (from El Gomhouria Co., Egypt) in the ratio (50:50) was added.

At 70ºC, the entire mixture was held on a magnetic stirrer. Finally, 2gm of silver nitrate (SIGMA-ALDRICH) was stirred into the mixture solution until it became homogeneous. Finally, after complete dissolution and mixing of silver and the other components, the mixture was kept in test tubes. The samples were irradiated via exposure to gamma radiation at an irradiation dose of 25kgy, the same procedures were utilized to synthesize PVP/Acrylamide/Acetic Acid containing silver nanoparticles without the addition of glycerol [29]. The sample was also exposed to a 25Kgy dosage of gamma radiation to be sterilized then cut into discs [30].

2.1.1. High - Resolution Transmission electron microscopy (HRTEM)

At 200 kV, the measurements were carried out using transmission electron microscope (JEOL, JEM 2100, Japan).

2.2. Effect of Silver Nanoparticles on bacteria

Evaluation of Silver Nanoparticles' Antibacterial Activity

The tested pathogenic bacteria included one Gram-positive (*Staphylococcus aureus* ATCC 6538) and three Gram-negative (*Escherichia coli* ATCC 8739, *Salmonella enterica* ATCC 14028, and *Shigella sonnei* ATCC 29930) Copad pharma Factory. The agar disc diffusion method was utilized to evaluate the antibacterial activity of two different types of silver nanoparticles, (AgNP1 and AgNP2). against tested organisms.

On nutrient agar plates, 100 µl of the test bacteria's actively growing broth cultures were spread out and given ten minutes to dry. Following a 48-hour incubation period at 37° C with sterile AgNP1 and AgNP2 discs (which were sterilized by exposure to gamma radiation) placed on the plates, the zone of growth inhibition surrounding the loaded discs was measured.

2.2.2. DNA Extraction and Inter Sample Sequence Repeat (ISSR) Analysis

DNA was extracted and purified from treated samples and negative control according to DNeasy 96 Mini Kit (QIAGEN, Germany). To estimate the DNA concentration; DNA samples (2µl) were subjected to a 1% agarose gel and compared to 10µl of a DNA size marker 0.1-3.0kb (pHi X 174/HaeIII digest, lambda DNA Hind III digest) (Cosmo Bio USA company). Using ISSR-PCR, eleven primers (Table 1) were utilized to analyze the genomes of treated and control bacteria [9].

For the PCR amplification, a PerkinElmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) was utilized. It was set up to complete 40 cycles following a 5-minute denaturation cycle at 94ºC. The steps in each cycle were as follows: 40 seconds at 94ºC for denaturation, 50ºC for annealing, and 60 seconds at 72ºC for elongation. The primer extension segment was extended to seven minutes at 72^{o} C in the final cycle. The amplification products were identified by electrophoresizing them in a 1.5% agarose gel with ethidium bromide (0.5 ug/ml) in 1X TBE buffer at 95 volts. [10] Utilizing a 100 bp DNA ladder (Thermo Scientific^{TM} GeneRuler 100bp Olus DNA Ladder,ready -to-use) as a molecular size standard, the product's dimensions were determined. To visualize the PCR products, they were exposed to UV light and captured on camera using the Gel Documentation System (BIO-RAD 2000).

Table 1. List of 11 primers used for ISSR amplification and their nucleotide sequences.

Primer	Sequence	
1-ISSR-1	5'-AGAGAGAGAGAGAGAGYC-3'	$(AG)_8YC$
2-ISSR-3	5'-ACACACACACACACACYT-3'	$(AC)_8YT$
3-ISSR-4	5'-ACACACACACACACACYG-3'	$(AC)_8YG$
4-ISSR-5	5'-GTGTGTGTGTGTGTGTYG-3'	$(GT)_8YG$
5-ISSR-7	5'-GACGATAGATAGATAGATA-3'	$GAC(GATA)_4$
6-ISSR-9	5'-GATAGATAGATAGATAGC-3'	$(GATA)_4GC$
7-ISSR-16	5'-TCTCTCTCTCTCTCTCA-3'	$(TC)_8A$
8-R-1	5'-CACACACACACACACACG-3'	$(CA)_8CG$
9-R-6	5'-TATATATATATATATAC-3'	$(TA)_8AC$
10-R-7	5'-CTCTCTCTCTCTCTCTT-3'	$(CT)_8T$
11-R-8	5'-GTGTGTGTGTGTGTGTC-3'	$(GT)_8C$

When comparing each sample's ISSR profile to the control profiles, the polymorphism that was seen included the band's disappearance and the emergence of a new band.

The band size acquired from ISSR analysis was determined regarding the existence of band (1) and the absence of it (0). The UPGMA technique in NTSYS-PC software was used to draw the dendrogram after the data was entered based on the molecular weight. GTS % = (1 − c/n) × 100) is the formula used to calculate genomic template stability (GTS %), where n is the total number of bands in the control sample and c is the average number of polymorphic bands found in each treated sample [2].

Using the following equation : BSI = 2S/(a + b), where a and b represent the first and second sample bands, respectively, and S is the number of bands shared by the two samples., the Band Sharing Index (BSI) was determined as a gauge of how similar two samples were: When the band sharing index (BSI) value is one, it indicates similarity between two samples; when the BSI value is zero, it indicates a complete difference between two samples [24].

3. Results

3.1. High - Resolution Transmission electron microscopy (HRTEM) measurement

According to HRTEM pictures (Fig. 1a and c), the silver nanoparticles had a variety of morphologies, including hexagonal, triangular, and cubic forms. (Thanh *et al*., 2019), The size distribution histogram of the silver nanoparticles immersed in PVP/ AAm/ Acetic acid, and glycerol is shown in Fig. 1(b). The silver nanoparticles ranged in size from 5 to 41 nm. Fig. 1(d) additionally displays the size distribution of the silver nanoparticles trapped in acetic acid and PVP/AAm . It was discovered that silver nanoparticles ranged in size from 23 to 47 nm. The HRTEM histogram yields size-controlled silver nanoparticles, which improve the stability and successful production of silver nanoparticles in the PVP/Acrylamide matrix.

3.2. Effect of PVP/Am/Acetic acid/ silver nanoparticles on pathogenic bacteria.

3.2.1. Analyzing Silver Nanoparticles' antibacterial efficacy

As shown in Table 2 and Fig. 2, the maximum inhibition zone (35 and 30 mm) was recorded for *Shigella sonnei* by AgNP1 or AgNP2 respectively, Comparing the antibacterial activity; the inhibition zones produced in the three Gram-negative bacteria by AgNP1 is larger than that recorded by AgNP2; while in *Staphylococcus aureus* AgNP1 recorded a smaller inhibition zone as compared with AgNP2 (Table 2).

In this work, all the obtained experimental data are expressed as the mean ± standard deviation (SD) for n = 3. The antibacterial results were analyzed by Student's t test, significance was set at p<.0.05.

3.2.2. Evaluation of potential toxicity of silver nanoparticles using Inter Simple Sequence Repeat (ISSR)-PCR

The silver nanoparticles (AgNP1 and AgNP2) effect on the genome of bacteria used in this study was examined using 11 ISSR primers. Electrophoretic bands obtained from amplification have been presented in Fig. (3). ISSR analysis showed polymorphism in band number and in the Genomic Template Stability (GTS) (Tables 3, 4, 5 & 6).

As shown in Tables (3), the eleven primers produced 35 polymorphic bands (disappeared and new band appeared) in *E. coli* treated with AgNP1(T1) and 29 polymorphic bands when treated with AgNP2 (T2), while *Salmonella enterica* recorded 18 and 17 polymorphic bands and *Shigella sonnei* recorded 34 and 32 in bacteria treated with AgNP1 and AgNP2 respectively (Table 4, 5). In contrast, Gram-positive bacteria *Staphylococcus aureus* treated with silver nanoparticles AgNP1 and AgNP2 recorded a maximum number of polymorphic bands as compared with that of Gram-negative and that treated with AgNP2 is higher (49 bands) than that recorded with AgNP1 (38).

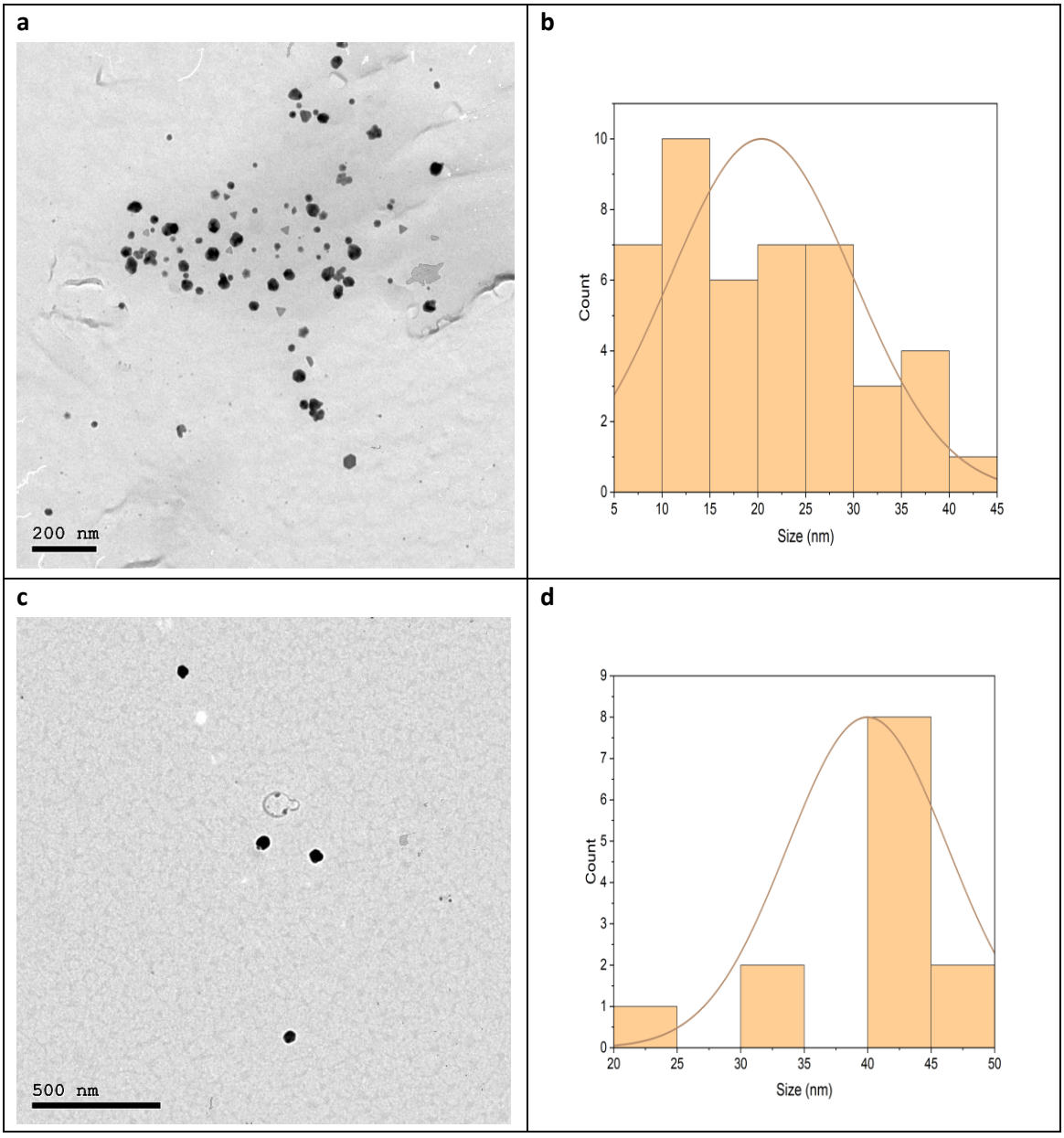


Fig. 1. (a) HRTEM image for silver nanoparticles embedded in PVP/ AAm/ Acetic acid/ Glycerol, **(b)** size distributions of silver nanoparticles for **(a)** sample**, (c)** HRTEM image for silver nanoparticles embedded in PVP/ AAm/ Acetic acid, and **(d)** size distributions of silver nanoparticles for **(d)** sample.

Table 2. Antibacterial activity. Values are mean of experiments performed in triplicate and data are expressed as mean ±SD

Test organism	Diameter of inhibition zone (mm)	
	AgNP1	**AgNP2**
1- *Escherichia coli*	24±0.8	23±1.4
2- *Salmonella enterica*	25±1.3	25±1.7
3- *Shigella sonnei*	35±2.16	30±0.8
4- *Staphylococcus aureus*	20±0.8	22±1.4

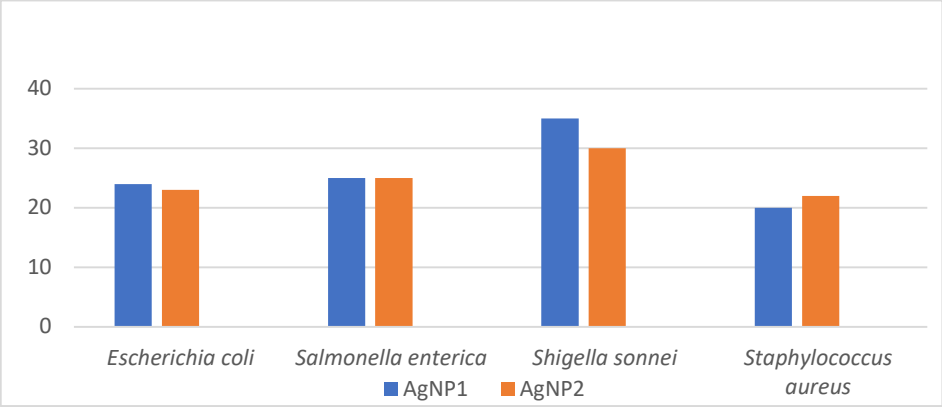
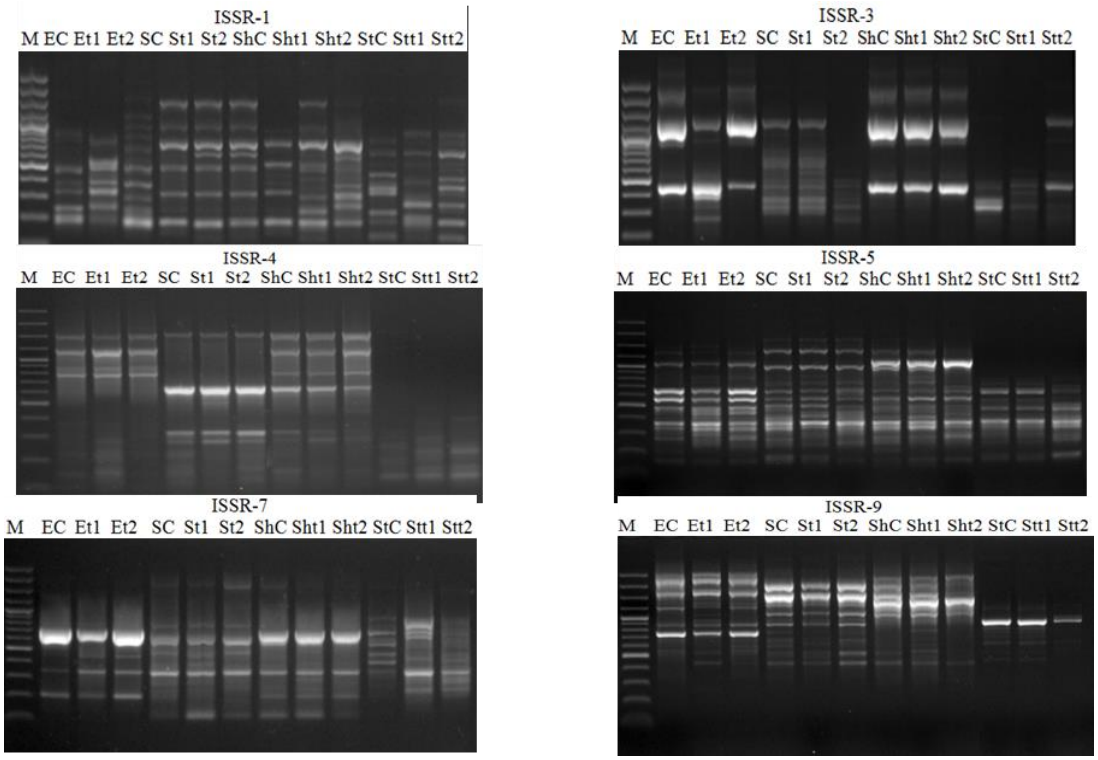


Fig. 2. Antibacterial activity of AgNPs



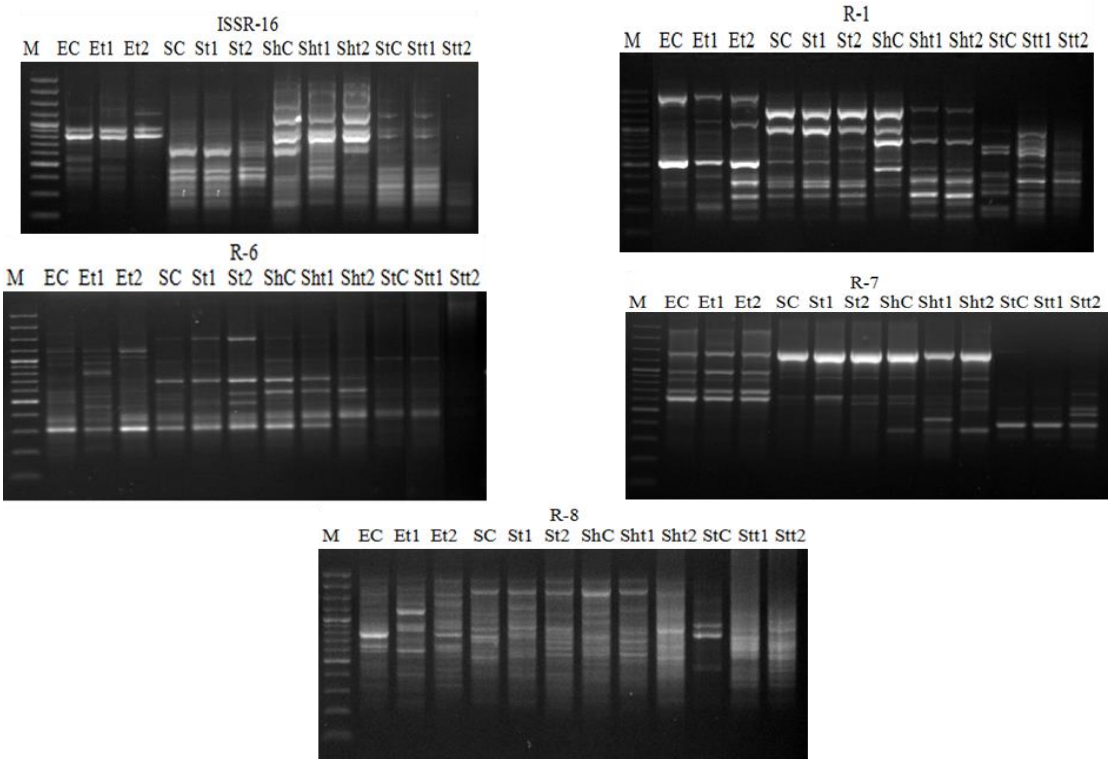


Fig. 3. The ISSR-PCR amplification of eleven primers produced electrophoretic bands. for three Gram-positive and one Gram-negative bacteria treated with silver nanoparticles where (M: Marker; EC: *E. coli* control; Et1: *E. coli* treated with AgNP1; Et2: *E. coli* treated with AgNP2; SC: *Salmonella enterica* control;St1: *Salmonella enterica* treated with AgNP1;St2: *Salmonella enterica* treated with AgNP2;ShC: *Shigella sonnei* control;Sht1: *Shigella sonnei* treated with AgNP1;Sht2: *Shigella sonnei* treated with AgNP2;StC:*Staphylococcus aureus* control;Stt1: *Staphylococcus aureus* treated with AgNP1;Stt2: *Staphylococcus aureus* treated with AgNP2)

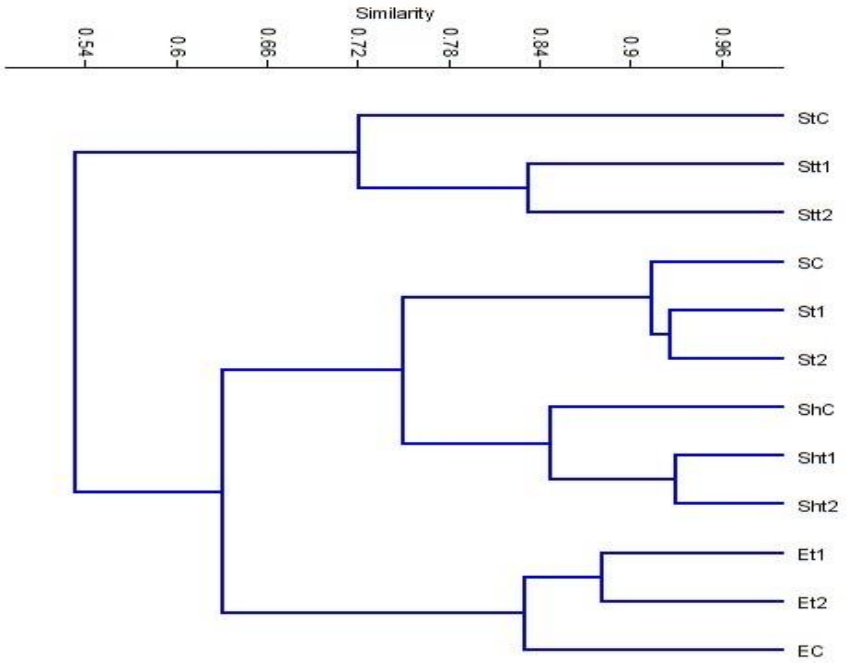


Fig. 4. DNA polymorphism based on ISSR-PCR analysis

Table 3. Polymorphism (No of disappeared and appeared band), genomic templet stability (GTS) and Band sharing Index (BSI) in *E. coli* treated with two types of silver nanoparticles (AgNP1and AgNP2) using ISSR-PCR.

Primer	Band No of control	Treatment of *E. coli* by AgNP1 (T1)			Treatment of *E. coli* by AgNP2 (T2)			Shared band between T1, T2
		No of band	Disappeared band (a)	Appeared band (b)	No of band	Disappeared band (a)	Appeared band (b)	
ISSR-1	8	6	2	1	11	-	3	7
ISSR-3	7	9	1	3	6	1	-	5
ISSR-4	6	7	-	1	6	-	-	6
ISSR-5	10	11	3	4	10	2	2	9
ISSR-7	5	6	-	1	6	-	1	6
ISSR-9	7	11	-	4	11	-	4	10
ISSR-16	7	7	-	-	7	1	1	6
R-1	9	9	2	2	11	2	4	8
R-6	8	9	2	3	10	-	2	8
R-7	6	8	-	2	8	-	2	8
R-9	13	15	1	3	15	1	3	15
total	86	98	11	24	101	7	22	88
(a +b)			35			29		
GTS%	100	59.4			66.3			
BSI		-			-			0.88

Table 4. Polymorphism (No of disappeared and appeared band), genomic templet stability and Band sharing Index in *Salmonella enterica* treated with two types of silver nanoparticles (T1and T2) using ISSR-PCR.

Primer	Band No of control	*Salmonella enterica* treated with AgNP1(T1)			*Salmonella enterica* treated with AgNP2 (T2)			Shared band between T1/T2
		NO of band	Disappeared band (a)	Appeared band (b)	No of band	Disappeared band (a)	Appeared band (b)	
ISSR-1	9	10	1	2	10	1	2	10
ISSR-3	9	9	-	-	6	4	1	5
ISSR-4	6	5	1	-	6	-	-	5
ISSR-5	14	13	3	2	14	1	1	12
ISSR-7	11	11	-	-	11	-	-	11
ISSR-9	11	10	2	1	11	-	-	9
ISSR-16	11	11	-	-	11	1	1	10
R-1	8	8	-	-	8	-	-	8
R-6	7	6	1	-	6	-	-	6
R-7	3	4	-	1	4	-	1	4
R-9	12	12	2	2	12	2	2	12
Total	101	99	10	8	99	9	8	92
a + b			18			17		
GTS%		82.2%			83.2%			
BSI		--			--			0.93

Table 5. Polymorphism (No of disappeared and appeared band), genomic templet stability and Band sharing Index in *Shigella sonnei* treated with two types of silver nanoparticles (T1and T2) using ISSR-PCR.

Primer	Band No of Control	*Shigella sonnei* treated with AgNP1 (T1)			*Shigella sonnei* treated with AgNP2 (T2)			Shared band between T1, T2
		No of band	Disappeared band (a)	Appeared band (b)	No of band	Disappeared band (a)	Appeared band (b)	
ISSR-1	8	13	1	6	13	2	7	12
ISSR-3	7	7	-	-	7	-	-	7
ISSR-4	9	9	-	-	9	-	-	9
ISSR-5	13	15	1	3	12	3	2	12
ISSR-7	7	9	-	2	8	-	1	8
ISSR-9	9	13	-	4	10	1	2	10
ISSR-16	13	12	3	2	15	-	2	12
R-1	8	9	1	2	9	1	2	8
R-6	9	7	2	-	7	2	-	6
R-7	5	7	-	2	6	-	1	6
R-9	13	14	2	3	13	3	3	13
Total No	101	115	10	24	109	12	20	103
a + b			34			32		
GTS%		66.3%			86.3			
BSI		--			--			0.92

Table 6. Polymorphism (No of disappeared and appeared band), genomic templet stability and Band sharing Index in *Staphylococcus aureus* treated with two types of silver nanoparticles (T1and T2) using ISSR-PCR.

Primer	Band No of Control	*Staphylococcus aureus* treated with AgNP1 (T1)			*Staphylococcus aureus* treated with AgNP2 (T2)			Shared band between T1, T2
		No of band	Disappeared band (a)	Appeared band (b)		Disappeared band (a)	Appeared band (b)	
ISSR-1	10	10	4	4	11	2	4	9
ISSR-3	4	3	1	-	6	1	3	3
ISSR-4	2	4	1	3	4	1	3	3
ISSR-5	8	8	-	-	9	1	2	7
ISSR-7	8	8	5	5	9	3	4	7
ISSR-9	6	6	-	-	4	2	-	4
ISSR-16	14	14	-	-	8	6	-	8
R-1	9	12	2	5	10	3	4	9
R-6	4	4	-	-	4	-	-	4
R-7	4	3	1	-	5	1	2	3
R-9	5	12	-	7	12	-	7	12
Total	74	84	14	24	82	20	29	69
a + b			38			49		
GTS %		48.6%			33.3%			
BSI		--			--			0.83

4. Discussion

The two copolymer of PVP/acrylamide hydrogels containing silver nanoparticles (AgNP1 and AgNP2) have remarkable effect on the growth of the four pathogenic bacteria used in this work as shown in Table 2 and Fig. 2. Various investigations demonstrated AgNPs' effectiveness against drug-resistant pathogenic bacteria. [17, 18]. Gram-negative bacteria are generally more susceptible to the effects of silver nanoparticles than Gram-positive bacteria because of the structural differences in the composition of their cell walls. The differences in structure, thickness, and cell contents can be used to explain why Gram-positive S. aureus exhibits low inhibition and Gram-negative E. coli exhibits strong inhibition [16]. The results of this study are consistent with those of Feng et al. (2000) [6], who demonstrated that Staphylococcus aureus has a thicker cell wall and more peptidoglycan molecules, which can give bacteria resistance to AgNPs. According to Pal et al. (2007) [20], Gram-negative bacteria have a thin cell wall and less peptidoglycan, making them an easy target for silver nanoparticles.

Silver nanoparticles can interact with the various bacteria cell surface and disturb its function. Numerous studies have shown that AgNPs adhere to and deposit on the cell surface of Gram-negative bacteria because of the lipopolysaccharides (LPS) present in their membrane [20]. The antibacterial effect seems to be conferred by the extremely small size of silver nanoparticles through which they destroy the cell membrane and create intracellular damage which renders bacteria more permeable. Using transmission electron microscopy (TEM), numerous pits can be observed at sites of damage inflicted by silver nanoparticles, [27]. Also, As shown by TEM, Raffi et al. (2008) [22] verified that exposure to silver nanoparticles for a short period of time causes a complete disruption of the E. coli cell membrane. Therefore, size plays a significant role, but it is not the only factor, in determining a particle's potential toxicity. Other characteristics of nanomaterials include their chemical composition, shape, surface structure, surface charge, aggregation, solubility, and the presence or absence of functional groups of other chemicals. In this study, the variations observed in ISSR-DNA profiles could be a factor for bacterial growth inhibition through the occurrence of mutations and alteration in the DNA sequence by silver nanoparticles.

Duran et al. (2016) [5] reported that when AgNPs interacts with DNA of the bacteria S. aureus, the cell division is interrupted in the primary stages. Li et al. (2011) [13] concluded that silver nanoparticles could pass through the barrier of bacterial cells, release ions of silver, and react with DNA. Silver ions intercalate between base pairs, disrupts the double helical structure of DNA, thus inhibiting replication and propagation of the cell and blocking the expression of some genes combined with the growth [3, 4, 24]. Al-Qurainy (2010) [1] suggested that ISSR assay can be used to appreciate how chemicals alter DNA structure in living organisms. The difference between the bands analyzed in the treated sample and the negative control sample indicated that the treated samples' DNA sequences had changed, which had an impact on how differently the primers annealed and how segments were amplified in PCR. An amplicon may be lost as a result of DNA template breakage because genetic rearrangements and point mutations can cause the loss or creation of new annealing sites, which can lead to the appearance or disappearance of new amplicons, respectively.

Radzig et al. (2013) [21] found that silver nanoparticles are responsible for damage and mutations in DNA repair genes in E. coli making mutant strains more susceptible to AgNPs. Sondi and Salopek-Sondi, (2004) [27] illustrated the effect of nanoparticles on the enzymes included in replication. However, an analysis of ISSR profiles revealed a decline in genetic template stability across all treatments. A high GTS value indicates that DNA alterations are less likely to occur in the genome, whereas a low GTS value suggests that there is a higher chance of alteration. In this study, Staphylococcus aureus as a model of Gram-positive bacteria recorded lower GTS values than that recorded with E. coli, Salmonella enterica and Shigella sonnei as models for Gram negative bacteria.

Data showed that AgNP1 was more effective on the genome of Gram-negative bacteria than AgNP2 while vice versa was recorded with Gram-positive bacteria. The data obtained with the band sharing index is matched with genetic template stability. Samples of Gram-positive bacteria treated with AgNP1 or AgNP2 showed minimum Band Sharing Index in comparison with Gram- negative. A band sharing index of 1 means that two samples are similar, while 0 denotes that two samples are totally dissimilar [24]. This data indicated that the silver nanoparticle has greater damage on genetic material of S. aureus and the two treatments were more different in their effect as compared with the other treatments.

5. Conclusion

Before suggesting the use of nanoparticles for therapeutic purposes, more research has to be done to confirm whether the bacteria become resistant to the particles and to examine the cytotoxicity of the particles.

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