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Molecular characterization of silver resistant *E. coli* strains isolated from patients suffering from diarrhea

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Silver nanoparticles (AgNPs) are considered a good alternative for antibiotics due to emerging Multidrug Resistance (MDR) crisis. Resistance to AgNPs is approximately limited among Gram-positive and Gram-negative pathogens. Low toxicity to human cells permits its safe use as a new antimicrobial with broad spectrum. Bacterial cells are used as a factory for AgNPs synthesis supplying a powerful antimicrobial with eco-friendly way. In this study, MDR *Escherichia coli* strains were recovered from patients attending Minia University hospital. Biogenic synthesis of AgNPs was performed using *E. coli* cells. Transmission Electron Microscopy (TEM) was used to characterize AgNPs size and shape. Antibacterial activity of AgNPs was tested against the MDR *E. coli* isolates. Screening for Sil and Omp genes was done using polymerase chain reaction (PCR). A total of 13 MDR *E. coli* bacterial culture supernatant isolates were recovered from patients under study. Biosynthesis of AgNPs was observed after addition of supernatant to $AgNO_3$ by color change from yellow to brown. TEM characterization indicated the presence of silver nanoparticles with 15-75 nm particle size range. Eleven of MDR *E. coli* isolates were sensitive to biogenic AgNPs under study. *SilB* and *SilE* genes were encoded by the two AgNPs-resistant *E. coli* isolates which were negative for *OmpF* and *OmpC* genes, respectively demonstrating the role of Sil efflux pump genes and porin deficiency in AgNPs resistance. As indicated, the emergence of silver resistance due to the wide spread of biocides including silver has become a great challenge for the treatment of different infections.

Key words: MDR *Escherichia coli*, *SilB*, *SilE*, silver resistance, *OmpF*, *OmpC*.

INTRODUCTION

Bacterial infections caused by *Escherichia coli* are the most common between hospitalized patients including septicemia, urinary tract infections, enteritis and neonatal meningitis (Allocati et al., 2013). Prevalence of antimicrobial resistance among clinical bacterial isolates is growing everyday representing a great medical challenge for microbiologists and health care professionals all over the world (Prestinaci et al., 2015). Trials for finding effective alternatives for routinely used antibiotics are being made to overcome antimicrobial resistance crisis. AgNPs are promising alternatives which possess great antibacterial and antiviral activity. Antibacterial activity of AgNPs was greater against Gram-negative bacteria than Gram-positive ones, due to

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attraction between negative charges on Gram-negative lipopolysaccharide and weak positive charge on AgNPs (Franci et al., 2015). Green synthesis using bacterial cells is considered a friendly approach for creating AgNPs in a more economic and safe way (Ghorbani, 2013). Bacterial cell enzymes such as nitrate reductase reduce Ag^+ ions to AgNPs, which are indicated by colorimetric reaction (Rajesh et al., 2014). Antibacterial activity of biosynthesized AgNPs has been screened against different Gram-negative isolates including *E. coli* indicating observable inhibitory activity (Abu-Zaid, 2016). Resistance to AgNPs can be correlated to decrease permeability either due to porin deficiency in Gram-negative cell wall or due to presence of *Sil* genes which encodes efflux pumps (Gupta et al., 1999). Porins (outer membrane proteins) are encoded by Omp genes. OmpF and OmpC are two major proteins of *E. coli* outer membrane, these proteins have a role in small hydrophilic molecules passive diffusion through bacterial membrane (Matsuyama et al., 1986). Sil efflux genes consist of SilRS (transcriptional regulatory system which consists of two components), this system controls SilE (protein binds to periplasm). Another components are two efflux pumps SilP (a P-type ATPase) and SilCBA (three protein chemiosmotic RND Ag (I)/H* exchange system) (Silver, 2003). In the present study, AgNPs were biosynthesized using *E. coli* cells, antibacterial activity of AgNPs against clinical MDR *E. coli* isolates, recovered from patients suffering from gastroenteritis, was studied using agar well diffusion method and the presence of *OmpC*, *OmpF*, *SilB* and *SilE*, *SilP*, *SilS* genes were screened among recovered isolates.

MATERIALS AND METHODS

Collection of samples

A total of 30 stool samples were collected from patients suffering from bacterial gastroenteritis attending department of accident and emergency in Minia University hospital (Minia, Egypt), samples were collected from March 2018 to April 2018.

Isolation of *E. coli* strains

Fecal samples were processed as follow: 3 g of fecal samples were mixed with normal saline, and centrifuged. The supernatants were discarded and the deposit was suspended in peptone water. Aliquots were used for the inoculation of MacConkey agar (Oxoid, UK), followed by an overnight incubation at 37°C. *E. coli* positive cultures were confirmed using biochemical tests (Himedia, India) (catalase positive, methyl red positive, indole positive, nitrate reduction positive and citrate negative) and the formation of metallic sheen on Eosin methylene blue agar.

Antimicrobial susceptibility testing

Amoxycillin/clavulanic acid, norfloxacin, azithromycin, cefoperazone levofloxacin, ceftriaxone, sulfamethoxazole/trimethoprime, cefuroxime, imipenem, tetracycline, cefipime, amikacin were used for testing antimicrobial activity against *E. coli* isolates. Disk method using Muller-Hinton agar plates was applied for antimicrobial susceptibility testing. By inoculating nutrient agar plates with suspension of inoculum and streaking of bacterial suspension using cotton swab. Antibiotic discs (Oxoid, UK) were applied after drying of inoculated nutrient agar plates. Diameters of inhibition zones were measured indicating sensitive or resistant isolates according to CLSI standards (CLSI, 2008).

Silver nanoparticles biosynthesis

E. coli culture was obtained from Microbiology and Immunology Department, Faculty of Pharmacy, Minia University. Nutrient broth was used to inoculate *E. coli* isolate. Supernatant was obtained by centrifugation of *E. coli* culture for 15 min at 5000 rpm and added to the flask of sterile aqueous $AgNO_3$ (1 mM). The reaction was performed for 10 min in bright conditions (Ghorbani, 2013).

Silver nanoparticle isolation and purification

By centrifugation of mixture of supernatant and $AgNO_3$ for 20 min at 10000 rpm, AgNPs were isolated. The AgNPs pellet was washed by centrifugation with sterile distilled water twice for 20 min at 1000 rpm, so the culture filtrate and excess silver ions were removed. Freeze drying was used to obtain AgNPs pellet as powder for further characterization and applications (Rajesh et al., 2014).

AgNPs characterization

Transmission electron microscope (TEM) (JEM1010, JEOL,Tokyo, Japan) was used to characterize shape and size of the isolated AgNPs. A nanoparticle solution drop was placed over carbon-coated copper grids and left for water evaporation. Then, size and shapes of AgNPs were examined (Rajesh et al., 2014).

AgNPs antibacterial activity

Antibacterial screening was performed using disc diffusion method by preparing nutrient agar and nutrient broth culture of tested isolates. Both nutrient agar and broth cultures were mixed together. The mixture was poured onto sterile petridishes. AgNPs discs were prepared by sterilizing filter paper discs in autoclave, dipping into $AgNO_3$ solution (10 µg/ml) and drying in air in sterile area. AgNPs discs were placed onto the nutrient agar after seeding with broth culture. After incubation for 24 h at 37°C, inhibitory activity of $AgNO_3$ discs was indicated by presence of inhibition zones around the discs (Rosoanaivo and Ratsimamamanga-Urvery, 1993; Malabadi et al., 2005). Data entry and analysis were all done using software called Statistical Package for Social Science (SPSS) (IBM, U.S.A).

DNA extraction

The DNA template was prepared by boiling of suspension of bacterial pellet for 10 min and directly used in the PCR assay. Genomic DNA was extracted from *E. coli* overnight culture by method described by Wilson (1987).

PCR primers and condition

PCR reaction was performed in a total of 50 µl reaction as follows: DNA extract (5 µl), each primer (50 pmole), Go Taq Green Master

Table 1. Primers in the study with corresponding amplification products.

Gene name	Sequence	Amplification product (bp)	References
OmpF	Forward: ACCTGGCAGCGAACTACG Reverse: AACATCACCGATACCTTCTACG	191	Vinson et al., 2010
OmpC	Forward: CAGGATGTGGGTTCTTTCG Reverse: GAAGTCAGTGTTACGGTAGG	162	Vinson et al., 2010
SilB	Forward: CAAAGAACAGCGCGT GATTA Reverse: GCTCAGACATTGCTGGCATA	233	Woods et al., 2009
SilE	Forward: GTACTCCCCCGGACATCACTAATT Reverse: GGCCAGACTGACCGTTATT	400	Percival et al., 2008
SilS	Forward: GGAGATCCCGGATGCATAGCAA Reverse: GTTTGCTGCATGACAGGCTAAAGACATC	1500	Percival et al., 2008
SilP	Forward: CATGACATATCCTGAAGACAGAAAATGC Reverse: CGGGCAGACCAGCAATAACAGATA	2500	Percival et al., 2008

mix (Promega) (25 µl). Conditions for *OmpC* and *OmpF* genes were: initial denaturation step at 94°C (3 min), 35 cycles of denaturation at 94°C (45 s), annealing of primers at 53°C for OmpC / 54°C for OmpF (45 s) and extension step at 72°C (1 min) then final extension at 72°C (5 min) (Vinson et al., 2010). Conditions for *SilB*, *SilS* and *SilP* genes were 95°C (5 min) and 30 cycles at 95°C (30 s), at 57°C (30 s) and 72°C (30 s) then final elongation at 72°C (5 min) (Losasso et al., 2014). Conditions for SilE gene were 94°C (5 min), 30 cycles at 94°C (30 s), 55°C (30 s), 72°C (3 min) then final elongation at 72°C (12 min) (Shutterlin et al., 2014). Agarose gel (2%) with ethidium bromide (Sisco Research Laboratories Pvt, Ltd., India) staining was used to analyze PCR products; gel document system was used to visualize DNA bands (Gel Doc 2000; Bio-Rad, USA)(Table 1).

RESULTS

Prevalence of isolates

In the present study, out of thirty stool samples, 13 (43.3%) *E. coli* isolates were recovered.

Antimicrobial susceptibility testing

Table 2 indicates that all *E. coli* isolates are resistant to most tested antimicrobial agents such as amoxycillin/ clavulanic acid, norfloxacin, azithromycin, cefoperazone, levofloxacin, ceftriaxone, sulfamethoxazole/ trimethoprime, cefuroxime and imipenem.

Silver nanoparticles synthesis

Silver nanoparticles formation were detected by the change of color of $AgNO_3$ solution after addition of bacterial supernatant to yellow, orange then brown (Figure 1).

Silver nanoparticle characterization

AgNPs characters were detected by TEM, the TEM micrographs of biogenic AgNPs showing that particles are spherical with size ranges from (15-75) nm (Figure 2).

Antibacterial activity of AgNPs against MDR *E. coli* isolates

Our study shows that out of 13 *E. coli* isolates tested, 11 (84.6%) were sensitive to AgNPs which indicated by presence of inhibition zone with a diameter ≥ 1.6 cm around prepared AgNPs discs (Table 3).

Screening of Omp and Sil genes

Figures 3a and b show that among 13 *E. coli* isolates tested, one isolate was positive for SilB gene and another was positive for SilE gene but No strain was positive for SilP or SilS genes. Figure 4 shows that 11 *E. coli* isolates tested were positive for OmpC and OmpF genes and only two isolates were negative for OmpF (SilB positive) and OmpC (SilE positive). All isolates were negative for SilS and SilP genes.

DISCUSSION

Silver was known for longtime by its antibacterial and antifungal activity. By increasing the incidence of antimicrobial resistance between clinical pathogens, silver use as AgNPs has found a great application in healthcare facilities such as wound dressings and antimicrobial agents (Shutterlin et al., 2012). Bacterial cells are found to supply a good factory for AgNPs synthesis (Ghorbani, 2013). In the present study, AgNPs

Table 2. Antimicrobial susceptibility of the tested *E. coli* isolates.

Isolate no.	Resistance pattern	Sensitivity pattern
1	AMC, NOR, AZM, CFP, LEV, CRO, SXT,CXM,IPM	TE, FEP, AK
2	AMC, NOR, AZM, CFP, LEV, CRO,SXT	TE, FEP, AK, CXM, IPM
3	AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM, IPM, TE	FEP, AK
4	AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM, IPM	TE, FEP, AK
5	AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM, IPM, AK	TE, FEP
6	AMC, NOR, AZM, CFP, LEV, CRO, SXT	CXM, IPM, AK, TE, FEP
7	AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM, IPM	AK, TE, FEP
8	AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM	IPM, TE, FEP, AK
9	AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM	IPM, TE, FEP, AK
10	AMC, NOR, AZM, CFP, LEV, CRO	SXT,CXM, IPM, TE, FEP, AK
11	AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM, IPM, TE	FEP, AK
12	AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM, IPM, AK	TE, FEP
13	AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM, AK	IPM, TE, FEP

AMC, Amoxycillin/clavulanic acid; NOR, Norfloxacin; AZM, Azithromycin; CFP, Cefoperazone; LEV, Levofloxacin; CRO, Ceftriaxone; SXT, Sulfamethoxazole/trimethoprime; CXM, Cefuroxime; IPM, Imipenem; TE, Tetracycline; FEP, Cefipime; AK, Amikacin.

Figure 1. Colorimetric reaction due to formation of AgNPs.

were synthesized biogenically from *E. coli* strain by addition of bacterial supernatant to $AgNO_3$ solution and the reaction color change to brown within 10 min in the light suggesting that nitrate reductase enzyme of *E. coli* reduced Ag^+ ions to AgNPs. Formation of brown color was suggested to be due to surface plasmon vibration excitation in AgNPs. Extracellular reduction by *E. coli* was applied for AgNPs synthesis (Ghorbani, 2013). *Lactobacillus acidophilus* culture was filtered and used for AgNPs synthesis by Ag^+ reduction at room temperature within one day time (Rajesh et al., 2014). Also, Nitrate reductase of *B. licheniformis* has a role in AgNPs synthesis (Kalimuthu et al., 2008). AgNPs isolated in this study and characterized by TEM micrographs showed spherical shape with size ranges from 15-75 nm. Smaller size AgNPs shows antimicrobial activity greater than larger ones (Rai et al., 2012). Also, shape of AgNPs is of great influence as rod-shaped and spherical AgNPs are less effective as biocidals against *E. coli* than triangular ones (Pal et al., 2007; Sharma et al., 2009). Particle size (4-50 nm) with spherical shape also indicated for AgNPs synthesized from *Lactobacillus acidophilus* (Rajesh et al., 2014). In another study, it was reported that AgNPs size ranges from 10-100 nm (Ghorbani, 2013).

The previous findings are in agreement with the results obtained in the present study as AgNPs were biosynthesized extracellularly with particle size ranges from 10-80 nm. As illustrated in our study, AgNPs discs showed inhibitory activity against 11 (84.6%) MDR *E. coli* clinical isolates. Gram-negative bacteria such as *Klebsiella pneumonia*, *E. coli* and Gram-positive such as *Staphylococcus aureus* and *Bacillus subtilis* are affected by antibacterial activity of AgNPs (Malabadi et al., 2012). Confirmation with high antimicrobial activity of biogenic AgNPs against MDR *E. coli* was reported (Abu-Zaid, 2016). It was indicated that *K. pneumoniae* was affected

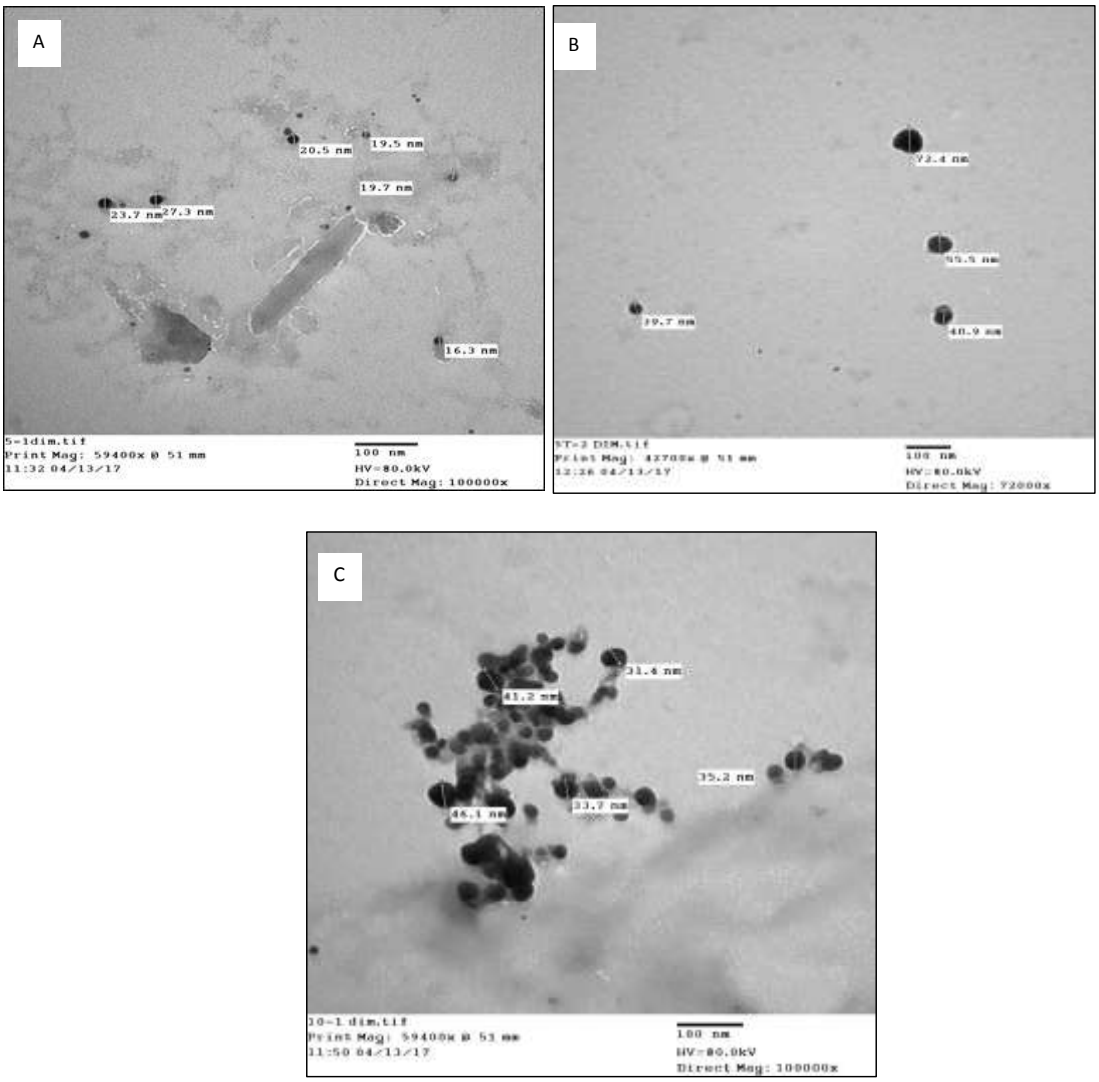

Figure 2. TEM micrographs of biogenic AgNPs.

Table 3. Antimicrobial activity of AgNPs against tested *E. coli* isolates.

***E. coli* isolated in the study**	**Zone of inhibition (cm)**
Isolate no. 1	1.6
Isolate no. 2	0
Isolate no. 3	1.6
Isolate no. 4	1.7
Isolate no. 5	1.8
Isolate no. 6	1.6
Isolate no. 7	1.7
Isolate no. 8	0
Isolate no. 9	1.7
Isolate no. 10	1.6
Isolate no. 11	1.6
Isolate no. 12	1.6
Isolate no. 13	1.6

Mean = 1.39, Standard deviation = 0.59.

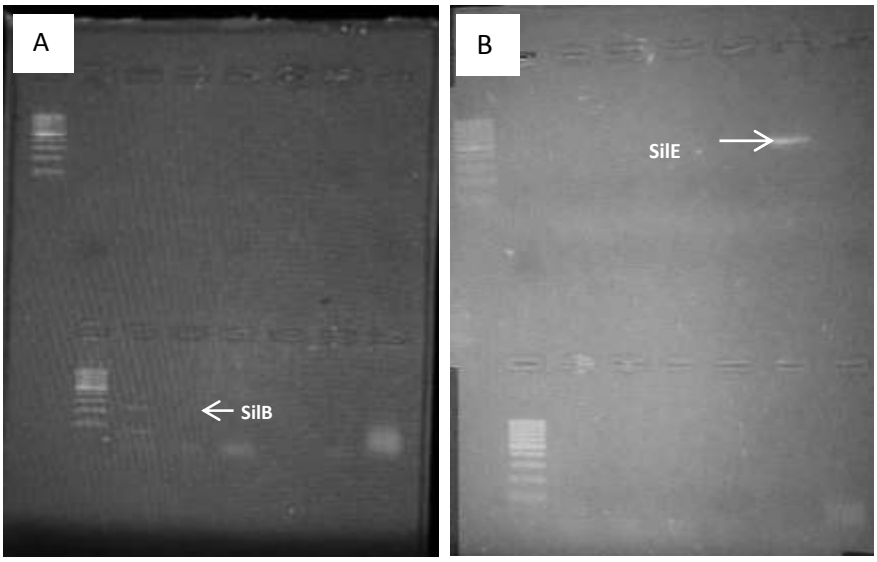

Figure 3. PCR products of SilB gene (A), all lanes are negative for SilB gene except lane 7 showed band of 233 bp and SilE gene (B), all lanes are negative for SilE gene except lane 2 showed a band of 400 bp.

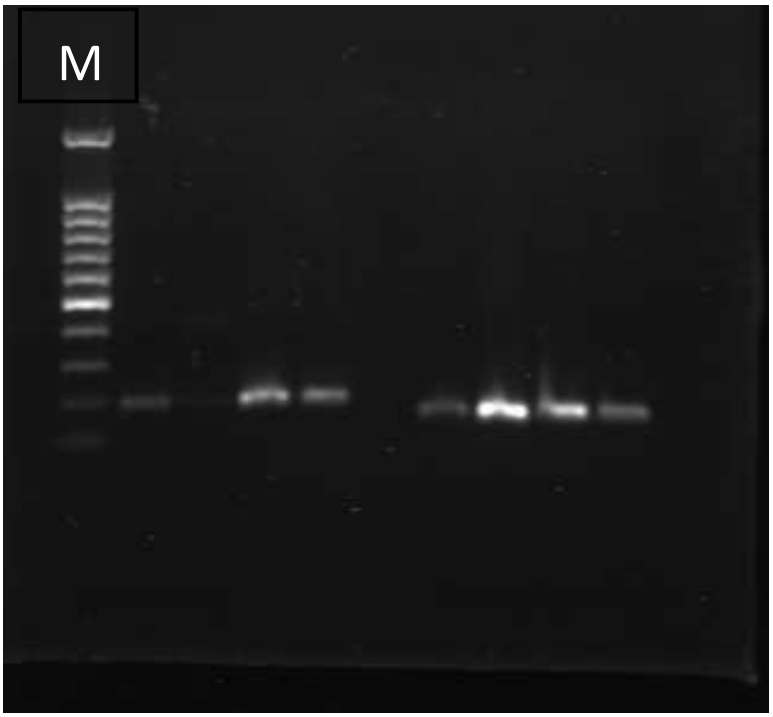

Figure 4. PCR products of OmpF (lanes 2-6 from left to right) and OmpC (lanes 7-11 from left to right), Lane 6: OmpF negative and Lane 11: OmpC negative.

by *Lactobacillus acidophilus*-synthesized AgNPs antibacterial activity (Rajesh et al., 2014). AgNPs antibacterial activity against *E. coli* was reported (Cunha et al., 2016; Li et al., 2010). Mode of action of AgNPs as antimicrobial may be attributed to free radicals generation, also after penetration of AgNPs into the cells, oxidative stress was reported (Hussain et al., 2006). Particle size of AgNPs is influential in its antibacterial

action as *E. coli* was killed by low concentration of 16 nm-size AgNPs, which was found inside bacteria and it is cell wall adherent (Raffin et al., 2008). Also, *E. coli* membrane disruption by 20 nm-size AgNPs was illustrated after few minutes' exposure indicating effect of large surface area of AgNPs on its antimicrobial efficiency (Raffin et al., 2008). Yeast, *Staphylococcus aureus* and *E. coli* were inhibited and killed by $AgNO_3$ and Ag^+ ions (Kim et al., 2007). Heat shock protein expression of *E. coli* was altered by short AgNPs exposure (Lok et al., 2006). Due to safe AgNPs use against human cells and effective low concentration, AgNPs are more applicable as antimicrobial agent (Abo-Neima and El-Khaly, 2016). In addition to antibacterial activity, also cells infected with HIV were killed due to AgNPs antiviral activity (Sun et al., 2005). It was found that combination of AgNPs with antibiotic increase its antibacterial activity (Ingle et al., 2009, Rathod and Ranganath, 2011). MDR *E. coli* and Streptococcus species showed great response to AgNPs antimicrobial action (Lara et al., 2010). All previous reports agree with our results as AgNPs showed good antibacterial activity against MDR isolates. In another finding, *E. coli* isolates showed no response to AgNPs indicated by absence of inhibition zone which is in a disagreement with results obtained in our study (Inbaneson et al., 2011). Our study shows that 11 isolates were positive for OmpC and OmpF genes as it confirms the presence of porin channels among 11 AgNPs-sensitive tested *E. coli* isolates. Also, absence of OmpC and OmpF genes is observed among AgNPs-resistant *E. coli* isolates. Ag+ ions enter the bacterial cell thorough porins and in case of porin protein mutation, *E. coli* becomes resistant to silver antibacterial activity (Li et al., 1997). When OmpF or OmpC porins are absent due to mutation, *E. coli* becomes 4-8 times less susceptible to the antibacterial activity of AgNPs (Radzig et al., 2013). The previous reports agree with our findings with respect to the role of Omp genes in *E. coli* response to antibacterial activity of AgNPs. The present study illustrated the absence of *SilB* and *SilE* genes among 11 tested *E. coli* isolates and only two isolates were positive for *SilB* and *SilE* genes for each. These two isolates were resistant to AgNPs antibacterial activity while other 11 isolates were AgNPs sensitive as confirmed by the absence of *Sil* genes. Bacteria resist silver by uptake decrease, cell membrane alteration and efflux pump that cause silver to be expelled out of cell (Silver and Phung, 2005). SilB is a fusion protein in periplasmic space which links SilA (pump protein present in inner membrane) to SilC (porin channel). SilB gene is an indicator for resistance of Gram-negative to silver (Silver et al., 2006). Whether in presence or absence of nanosilver, SilB gene is constitutively expressed among *Salmonella senftenberg* (Losasso et al., 2014). SilE gene was common among *E. coli* isolates producing CTX-M after exposure to silver (Shutterlin et al., 2014). The previous findings are in agreement with our results as Sil operon plays a role in AgNPs resistance. As illustrated in our study, one *E. coli* isolate was found negative for OmpF and positive for SilB and another *E. coli* isolate was found negative for OmpC and positive for SilE, these two isolates were AgNPs resistant confirming the role of efflux pump encoded by Sil gene and the effect of porin absence in *E. coli* resistance to AgNPs. In another report, Sil genes were not correlated to resistance of *E. coli* to silver (Shutterlin et al., 2012) which contradicts with the findings of the present study.

Conclusion

Although silver resistance was not frequent among bacterial isolates for long time, the presence of Sil genes and absence of Omp genes have a great influence in *E. coli* response to AgNPs antibacterial activity indicating that silver resistance among MDR isolates is a possible behavior which threatens the power of silver as a strong old biocidal.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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