



In Vitro Investigation of the Antibacterial Effect of Silver Nanoparticles on ESBL-producing *E. coli* and *Klebsiella* spp. Isolated from Pet Animals

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ABSTRACT

Despite the presence of modern antibacterial drugs, bacterial infections are still a major threatening problem due to the enormous increase in multi-drug-resistant bacteria. Nanoparticles have been extensively used as an applicable and safe alternative to antibiotics. The present study aimed to explore the inhibitory effect of silver nanoparticles on Extended Spectrum Beta lactamase (ESBL) producing *E. coli* and *Klebsiella* spp. in vitro as well as their effect on the expression of antibiotic resistance genes. Different samples (i.e., wound swabs, Fecal swabs, and urine samples) were collected from dogs and cats. Phenotypic and molecular identification, antibiotic susceptibility testing, and double-disk synergy test were carried out for the identification of ESBL producing *E. coli* and *Klebsiella* spp. Silver nanoparticles were tested for their in vitro antibacterial potential and there were reports of their minimum inhibitory concentration and minimum bactericidal concentration. Moreover, the effect of silver nanoparticles on the expression of antibiotic resistance genes (i.e., *blaTEM*, *blaSHV*, and *blaCTX*) was assessed as well as their effect on the structural integrity of the bacterial cells using Scanning Electron Microscope (SEM). Results revealed that 23 isolates (19.16%) (*E. coli*=17, *Klebsiella* spp.=6) were confirmed as ESBL producing. Silver nanoparticles indicated a promising antibacterial effect where the minimum inhibitory concentration of AgNPs for ESBL producing *E. coli* was measured as 0.31 mg/ml, and 0.62 mg/ml for ESBL-producing *Klebsiella* spp., while the minimum bactericidal concentration of ESBL-producing *E. coli* and *Klebsiella* spp. was reported as 0.15 mg/ml and 0.3 mg/ml, respectively. Consequently, the expression of antibiotic resistance genes was downregulated in both bacteria species and there was a noticeable toxic effect of AgNPs on *E. coli* and *Klebsiella* spp. cells which was investigated using SEM. It can be concluded that silver nanoparticles have a promising antibacterial activity and could be considered an applicable alternative for the control of ESBL producing bacteria.

Keywords: *E. coli*, ESBLs, *Klebsiella* spp., Pets, Silver nanoparticles

INTRODUCTION

In the current decade, the search for antibiotic alternatives has become one of the most quintessential issues due to the massive expansion of antibiotic resistance. The usage of silver nanoparticles has been widely recognized due to their known bactericidal as well as bacteriostatic actions against different types of bacterial and fungal pathogens (Saeb et al., 2014). Silver nanoparticles (AgNPs) have a broad-spectrum antimicrobial effect due to their large surface area providing the chance for better contact with microbes (Li et al., 2010). Furthermore, silver nanoparticles have a lower propensity to induce microbial resistance than other antimicrobial agents (Ansari et al., 2014). It is also worth mentioning that these nanoparticles have a non-toxic effect on the human at low concentrations (Bindu et al., 2015). They are characterized by their powerful antioxidant and antibacterial effect because of bioactive molecules on the exterior surface of silver nanoparticles (Keshari, 2020).

Extended Spectrum β -Lactamase producing bacteria (ESBL) are types of bacteria that show resistance to several types of antibiotics through the hydrolysis of the β -lactam ring of antibiotics (Kizilca et al., 2012) and they can transfer resistance to penicillins, third-generation cephalosporins, and monobactams (Ejaz et al., 2011). Moreover, most of them are not inhibited by other non- β -lactam antibiotics since the resistance encoding genes of other antibiotic classes can also be carried by the plasmids containing the ESBL-encoding genes (Alyamani et al., 2017; Fan et al., 2014). *Escherichia coli* and *Klebsiella* spp. remain the major ESBL-producing microorganisms isolated worldwide. They are considered the most emerging Extended-spectrum β -lactamase (ESBL) with a serious effect on the community (Devrim et al., 2011). The CTX-M-type enzymes are the largest ESBL groups spreading globally, followed by TEM and SHV groups (Sukmawinata et al., 2020). Researchers from different countries have successfully isolated and identified ESBL-producing bacteria from different types of samples, including isolates from human in Bahrain (Shahid et al., 2014), different samples (e.g., fecal swabs, animal feeds, water, and excreta) from different animals (e.g., dogs, cats, sheep,

goat, chickens, turkey, ducks, and human, (Okapara et al., 2018), isolates of urine samples from dogs and cats in USA and Switzerland (Thungrat et al., 2015; Zogg et al., 2018), also isolates of fecal swabs from dogs and cats in Newzeland (Karkaba et al., 2019). Nanoparticles are known mainly by their direct action on the bacterial cell wall which is different from other antibiotic resistance mechanisms and needs no penetration of bacterial cells (Wang et al., 2019). Subsequently, they are less predisposed to develop resistance, compare to antibiotics. AgNps have proved a good antibacterial and antioxidant activity against *E. coli* and *Klebsiella pneumoniae* isolates (Khan et al., 2020). Moreover, it was demonstrated that AgNPs have a powerful toxic action on ampicillin resistant *Klebsiella Pneumoniae* genes and bacterial proteins as well as bacterial membrane damage and oxidative stress (Hamida et al., 2020). Therefore, this study aimed to explore the inhibitory effect of silver nanoparticles on ESBL-producing *E. coli* and *Klebsiella* spp. in vitro as well as their effect on the expression of resistance genes. In doing so, the phenotypic and molecular identification of ESBL producing *E. coli* and *Klebsiella* spp. was carried on different samples collected from dogs and cats living in various places in Egypt. The bactericidal activity of Ag nanoparticles was assessed via different microbiological and molecular techniques.

MATERIALS AND METHODS

Ethical approval

The study was conducted according to ethical guidelines approved by the Faculty of Veterinary Medicine, Cairo University. There were no experiments applied to human participants.

Samples collection and preparation

A total of 120 samples were collected from diseased dogs (n=55) and cats (n=65). All samples were collected according to the guidelines of the Institutional Animal Care and Use Committee at Cairo University and approved by Vet-CU-IACUC (Vet CU 16072020198), Cairo, Egypt. Written consent was obtained from the animal owners after they were informed on the use of their animal samples in the study. Samples included fecal swabs (n=61), wound swabs (n=17), and urine samples (n=42). Samples were collected from Al-Shaab Veterinary Hospital, Surgery and Medicine Departments at Faculty of Veterinary medicine, Cairo University as well as animal laboratories in Cairo and Giza from March to December 2019. Samples were collected from animals suffering from gastrointestinal tract disturbances, and acute or chronic cystitis. In addition, the wounded animals had certain medical conditions and could provide no appropriate response to the prescribed antibiotics (Huber et al., 2013). All investigated samples were collected under aseptic conditions and safety precautions. Samples were directly inoculated into 9 ml of sterile physiological saline (Okapara et al., 2018). Urine samples were collected from each case via catheter and urine was collected from the distal part under aseptic conditions by the collection of the midstream urine sample (Cystocentesis) as reported by Huber et al. (2013). Samples were appropriately labelled and transported without delay to the laboratory and processed immediately.

Phenotypic characterization and Antibigram testing for *E. coli* and *Klebsiella* spp. isolates

Wound and fecal swabs were inoculated onto MacConkey agar (Oxoid) supplemented with ampicillin (100 mg/L; Mac-AMP100, Oxoid) according to Okapara et al. (2018). Urine samples were centrifuged, and the sediment was inoculated directly on MacConkey agar (Oxoid). All inoculated plates were incubated at 37°C for 18-24 hours and examined for bacterial growth. Both lactose fermenter colonies and late lactose fermenter colonies were selected for further examinations. The purified isolates were finally confirmed biochemically with citrate, oxidase, indole, catalase, Voges Proskauer, methyl red, urease, and triple sugar iron (TSI) tests according to Cruickshank et al. (1975). All isolates were tested for their susceptibility to different antimicrobial drugs and antibiotics (Table 1). The antimicrobial susceptibility test was performed using the disc diffusion method (Kibry-Bauer method) on Muller-Hinton agar plates (Oxoid) and the interpretation was performed based on CLSI (2018).

Doubled-disc synergy test

ESBL production was identified using Double Disk Synergy Test (DDST) according to Iqbal et al. (2017). Three antibiotics were used for DDST ceftriaxone (30µg), Amoxicillin-clavulanic acid (20/10µg), and ceftazidime (30µg, Oxoid). Discs were placed at a distance of 1.5cm. ESBL positive organism are showing development of the inhibition zone towards the clavulanate disc at 37°C after 24-hours-incubation.

Molecular characterization of ESBL-genes in *E. coli* and *Klebsiella* spp. isolates

Extraction of DNA was performed using QIAmp DNA Mini Kit instructions (QIAGEN, Germany). Specific primers were used for the amplification of *blaTEM*, *blaSHV*, and *blaCTX* genes (Table 2). The preparation of the PCR Master Mix was performed according to Emerald Amp GT PCR Master Mix (Takara). The reaction mixture consisted of 12.5µl Emerald Amp GT PCR Master Mix (2x premix), 4.5 µl PCR grade water, 1µl of each primer in the concentration

of (20 pmol), 6µl of Template DNA, and leading to a total of 25µl. The cycling condition was like that used in (Hasman et al., 2005). The ladder was mixed gently by pipetting up and down, and 6 µl of the required ladder was directly loaded. The PCR products were resolved by electrophoresis on an agarose gel according to Sambrook et al. (1989) with some modification.

Table 1. Antibiotics used in antimicrobial susceptibility test and their resistance pattern against *E. coli* and *Klebsiella* spp. isolates

Antibiotic	Disc content	Resistance pattern of <i>E. Coli</i>			Resistance pattern of <i>Klebsiella</i> spp.		
		Sensitive (%)	Intermediate (%)	Resistant (%)	Sensitive (%)	Intermediate (%)	Resistant (%)
Aminoglycosides							
Amikacin	30 µg	89	5	6	86	6	8
Gentamycin	10 µg	53	18	29	72	0	28
Streptomycin	10 µg	47	0	53	28	20	52
Kanamycin	10 µg	31	13	56	60	12	28
Cephalosporins (1st generation)							
Cephalexin	30 µg	8	0	92	18	6	76
Cephalosporins (3rd generation)							
Cefotaxime	30 µg	9	4	87	52	0	48
Ceftazidime	30 µg	19	0	81	36	4	60
Ceftriaxone	30 µg	23	6	71	38	22	40
Miscellaneous antibiotics							
Chloramphenicol	10 µg	49	27	34	45	31	24
Nitrofurantoin	300 µg	69	13	18	58	24	28
Other β lactam							
Aztreonam	30 µg	41	6	53	66	10	24
Penicillins							
Ampicillin	10 µg	0	0	100	0	0	100
Amoxicillin	(20/10) µg	37	0	63	32	8	60
Clavulanic acid							
Quinolones							
Ciprofloxacin	5 µg	71	11	18	88	0	12
Nalidixic acid	30 µg	68	3	29	92	0	8
Tetracyclines							
Tetracycline	30 µg	34	6	60	42	22	36

Table 2. Oligonucleotide primers and probes used in PCR and SYBR Green real-time PCR

Gene	Primer sequence (5'-3')	Amplification size	Reference
<i>bla_{TEM}</i>	ATCAGCAATAAACCAGC	516 bp	Colom et al. (2003)
	CCCCGAAGAACGTTTTTC		
<i>bla_{SHV}</i>	AGGATTGACTGCCTTTTTTG	392 bp	
	ATTTGCTGATTCGCTCG		
<i>bla_{CTX}</i>	ATG TGC AGY ACC AGT AAR GTK ATG GC	593 bp	Archambault et al. (2006)
	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		
<i>gyrA (Klebsiella spp.)</i>	CGC GTA CTA TAC GCC ATG AAC GTA	-	Brisse and Verhoef (2001)
	ACC GTT GAT CAC TTC GGT CAG G		
<i>16S rRNA (E. coli)</i>	GCTGACGAGTGGCGGACGGG	-	Tivendale et al. (2004)
	TAGGAGTCTGGACCGTGCT		

In vitro assessment of the antibacterial effect of Ag NPs suspension

Silver nanoparticles powder was purchased from the National Research Center, Egypt. The dispersion process was done using an ultrasonic processor (Cole-Parmer instruments, Illinois U.S.A.). For nanofluid preparation, 10 mg of Ag NPs of average size 58 nm were dispersed in 1ml of sterile Muller Hinton broth and sonicated for 5 minutes at 20000 HZ frequency from 3 to 5 times to avoid aggregation (Tayel et al., 2010). Pure colonies from both *E. coli* and *Klebsiella* spp.

isolates were picked up and suspended in Muller Hinton broth. Suspensions were adjusted to match McFarland standard 0.5 (1.5×10^8 CFU /ml) to be ready for antimicrobial testing. The tests were adapted according to CLSI (2018). The minimum inhibitory concentration (MIC) of AgNPs was determined in a sterile 96-well microtiter plate. The 100 μ L of sterile muller Hinton broth was pipetted into the well No.1 through No.12 of the column. In the next step, 100 μ L of the Ag NPs suspension (10mg/ml) were added into well No.1 of column (A), to reach a total volume of 200 μ L with a concentration of (5 mg/ml). Two-fold serial dilution was applied starting with the previously mentioned concentration. Later, 100 μ L of the *E. coli* suspension (McFarland 0.5) was added to the wells of the column, and then the initial concentration changed to 2.5 mg/ml. Well No.11 served as a negative growth (sterility control) containing Ag NPs suspension plus sterile broth only while well No.12 was served as a positive bacterial control containing broth plus the bacterial inoculum only. These steps were repeated with *Klebsiella* spp. in another microtiter plate. The microtiter plates were incubated at 37 °C for 24 hours in a shaker incubator. The lowest concentration of Ag NPs in the series inhibiting the growth of the bacteria in vitro was taken as the MIC. For MBC determination, 50 μ L from each well was spread on MacConkey agar plates and incubated at 37°C for a further 48 hours. Growth-free plates validated that the used concentration inhibited bacterial growth. These tests were performed in triplicate.

Assessment of the inhibitory effect of AgNPs on the expression of resistance genes in *E. coli* and *Klebsiella* spp. using SYBR Green RT- PCR

The effect of sub-MIC dose (1/2) of AgNPs on the expression of *bla*TEM, *bla*SHV, and *bla*CTX genes was studied in the presence of 16s rRNA for *E. coli* and *gyrA* for *Klebsiella* spp. as housekeeping genes (Brisse and Verhoef, 2001; Tivendal et al., 2004). The RT-PCR procedure was performed in Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Egypt. Extraction of RNA was performed according to RNeasy Mini Kit instructions (QIAGEN, Germany, GmbH). Oligonucleotide primers and probes used in SYBR Green real-time PCR are shown in Table 2. PCR Master Mix QuantiTect SYBR Green PCR Kit was used. The reaction mixture consisted of 12.5 μ L 2x QuantiTect SYBR Green PCR Master Mix, 0.25 μ L Revert Aid Reverse Transcriptase (ThermoFisher, 200 U/ μ L), 0.5 μ L of each primer (20 pmol), 8.25 μ L RNase Free Water, 3 μ L Template RNA, leading to a total of 25 μ L. The cycling conditions were performed according to previous studies (Brisse and Verhoef, 2001; Colom et al., 2003; Tivendale et al., 2004; Archambault et al., 2006). Amplification curves and CT values were determined by the strata gene MX3005P software. To estimate the variation of gene expression of the different samples, the CT of each sample was compared with that of the control group according to the " $\Delta\Delta$ Ct method CT" stated by Yuan et al. (2006) and samples were tested in triplicates. The dissociation curves of different samples were compared to exclude false-positive results.

Evaluation of morphological changes in *E. coli* and *Klebsiella* spp. upon their interaction with silver nanoparticles

Samples included untreated samples (control) and AgNPs treated *E. coli* and *Klebsiella* spp. colonies. The samples were fixed by glutaraldehyde 2.5% and dehydrated by the serial dilution of ethanol with agitation using an automatic tissue processor (Leica EM TP, Leica Microsystems: Austria). In the next step, they were dried using CO₂ critical point drier (Model: Audosamdri-815, Tousimis; Rockville, Maryland, USA). The samples were coated by a gold sputter coater (SPI-Module, USA). They were examined by Scanning electron microscopy (Model: JSM- 5500 LV; JEOL Ltd –Japan) using a high vacuum mode at the Regional Center of Mycology and Biotechnology, Cairo, Egypt.

Statistical analysis

Statistical analysis was performed using R-programme. One-way ANOVA was run to evaluate the statistical significance between the control and treated samples. *P-value* less than 0.05 was considered statistically significant.

RESULTS

Phenotypic identification of *E. coli* and *Klebsiella* spp. isolates

Out of 120 samples, *E. coli* and *Klebsiella* spp. were detected in 62 (51.6%) and 25 (20.8%) cases, respectively. On MacConkey agar, *E. coli* appeared as medium-sized, smooth, round, lactose-fermenting colonies, pink to red with bile salt precipitate surrounding the colonies. On the other hand, *Klebsiella* spp. was observed as medium-sized, pink, lactose- fermenting, round, shiny, and mucoid colonies. Microscopic examination of Gram-stained pure colonies of *E. coli* and *klebsiella* spp. isolates were gram-negative rod-shaped bacteria. Isolates were confirmed biochemically. *E. coli* was negative in urease, oxidase, Voges Proskauer (vp), citrate tests while positive for catalase, methyl red (MR), Indole tests and A/A with gas production and negative H₂S production for TSI test. *Klebsiella* spp. was positive in urease, oxidase, Voges Proskauer, catalase and citrate test and A/A with gas production and negative indole, Methyl red, and H₂S production for TSI test.

Antimicrobial susceptibility testing

Using different groups of antibiotics (Table 1), the antibiogram was assessed for *E. coli* and *Klebsiella* spp. isolates. Ampicillin showed the highest percentage for resistance in both *E. coli* and *Klebsiella* spp. as shown in the resistance pattern (Figure 1). The most prominent groups in resistance pattern were Cephalosporins first and third generation represented by Cephalexin (92%, 76%), Cefotaxime (87%, 48%), Ceftazidime (81%, 60%), Ceftriaxone (71%, 40%) for *E. coli* and *Klebsiella* spp., respectively. Moreover, Amoxicillin Clavulanic acid indicated 63% resistance for *E. coli* isolates and 60% for *Klebsiella* spp. The overall result of the antibiogram revealed that about 29 isolates (*E. coli* =20, *Klebsiella* spp.=9) were suspected to be ESBL producing isolates.

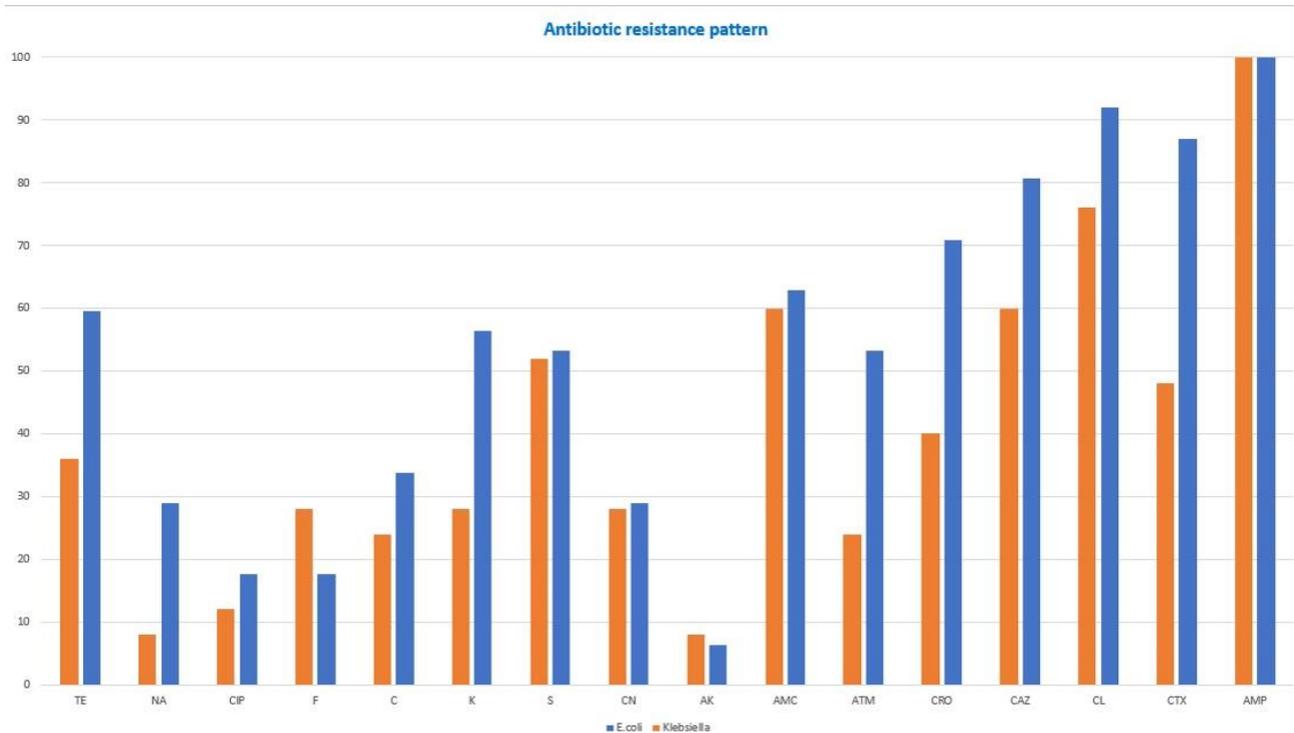


Figure 1. Antibiotic resistance pattern of the used antibiotics against *E. coli* and *Klebsiella* spp. isolates

Double-disc synergy test method

Detection of suspected isolates by DDST revealed that 23 isolates (*E. coli*=17, *Klebsiella*=6) were ESBL producing isolates. The number of isolated ESBL producing *E. coli* and *Klebsiella* spp. for each type of sample is demonstrated in Table 3.

Molecular detection of ESBL -encoding genes

PCR screening of genes encoding ESBL revealed the presence of *blaCTX*, *blaSHV*, and *blaTEM* genes in all the tested isolates except one *Klebsiella* spp. isolate which did not harbor *blaCTX* gene (Figure 2).

In vitro evaluation of the antibacterial effect of Ag NPs

Scanning Electron Microscopy of AgNPs (Figure 3) revealed that Ag NPs were spherical with the average size of the 58 nm. The MIC of AgNPs tested for ESBL-producing *E. coli* was 0.31 mg/ml, and 0.62 mg/ml for ESBL-producing *Klebsiella* spp. The minimum bactericidal concentration (MBC) of ESBL-producing *E. coli* and *Klebsiella* spp. was 0.15 mg/ml and 0.3 mg/ml, respectively.

Effect of sub MIC concentration of AgNPs on the expression of resistance genes.

Expression of *blaTEM*, *blaSHV*, and *blaCTX* genes was downregulated with sub-MIC doses of AgNPs (150 µg/ml for *E. coli* and 310 µg/ml for *Klebsiella* spp.), compared to untreated sample as shown in (Figure 4).

Effect of silver nanoparticles on the integrity of cells

The SEM images of untreated (control) cells showed healthy cells with clear unpenetrated cell membranes while treated cells showed cell membrane damage due to the adherence of AgNPs with the bacterial cell membrane and penetration into the cells causing cell death (Figure 5).

Table 3. Prevalence of ESBL producers from dogs and cats in Egypt

Origin	No. of samples with ESBL producer (%)		
	EC	KS	Total
Wound swabs (n=17)	-	2 (11.7)	2 (11.7%)
Fecal swabs (n=61)	10 (16.4%)	1 (1.6%)	11 (18%)
Urine samples (n=42)	7 (16.7%)	3 (7.1%)	10 (23.8%)
Total (n=120)	17 (14.16%)	6 (5%)	23 (19.6%)

EC: Escherichia coli; KS: *Klebsiella* spp.

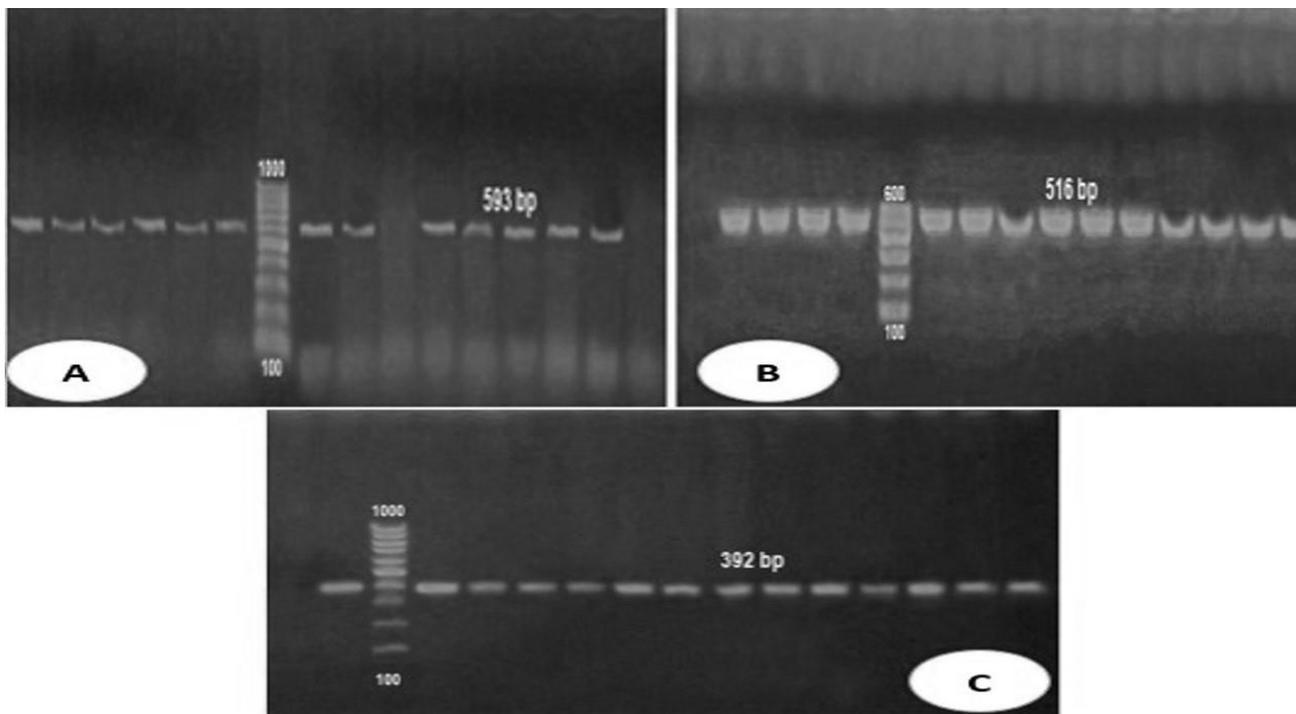


Figure 2. Agarose gel electrophoresis of A- *blaCTX* gene (Amplicon size 593 bp), B- *blaTEM* gene (Amplicon size 516 bp), C-*blaSHV* gene (Amplicon size 392 bp) Ladder [Gelpilot100 bp plus ladder (Qiagen, 100-1500 bp)]

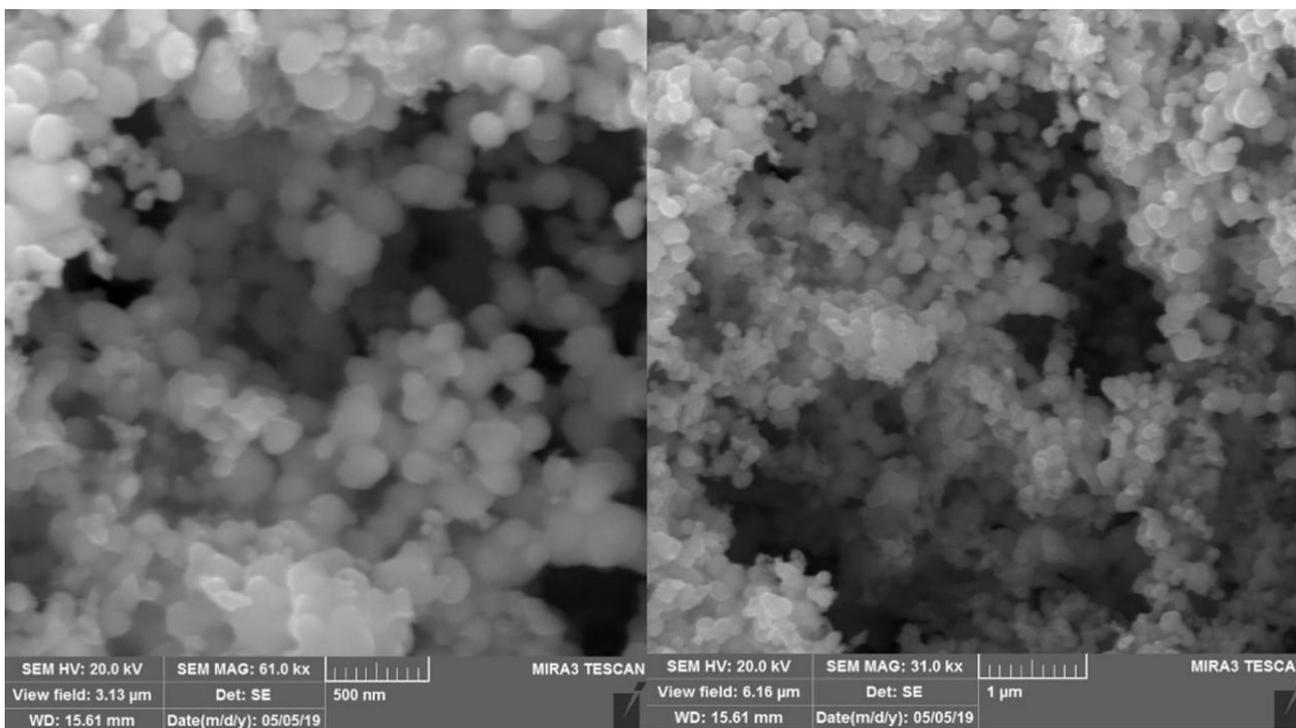


Figure 3. SEM of AgNPs revealed that Ag NPs are spherical in shape and the average size is 58 nm.

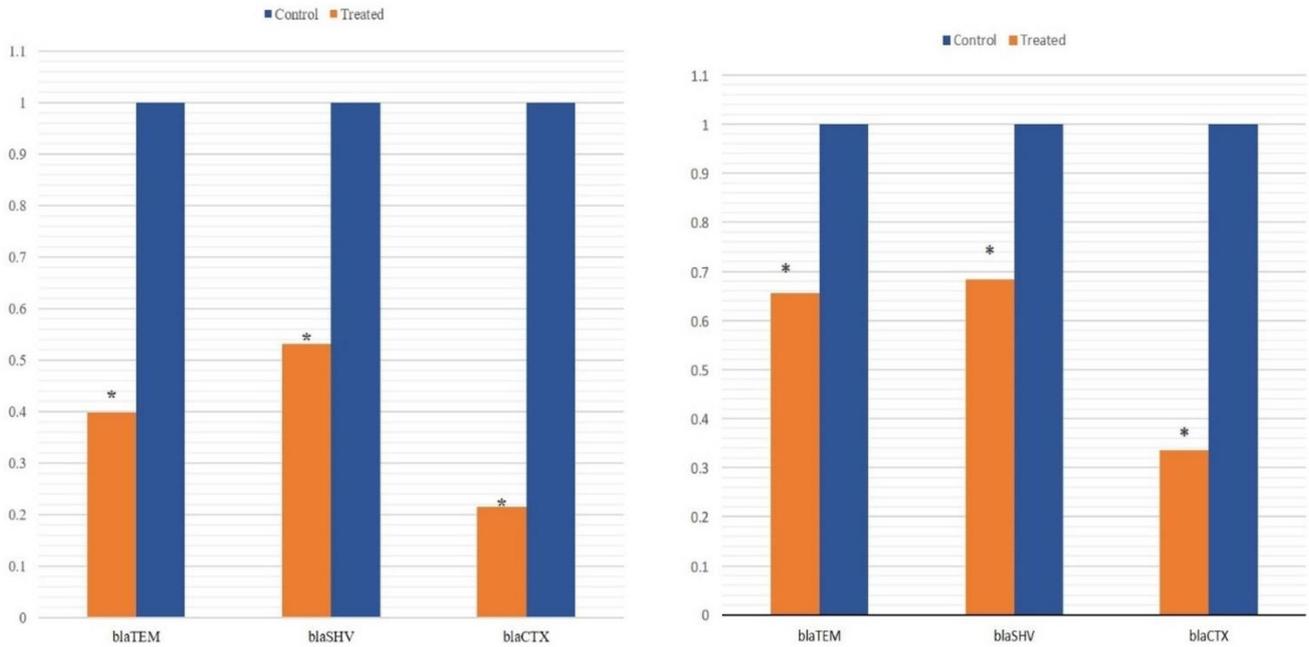


Figure 4. Effect of Silver nanoparticles on the antibiotic resistance genes expression in Left *E. coli*, Right *Klebsiella* spp. Stars indicate significant difference between control and treated samples for each gene.

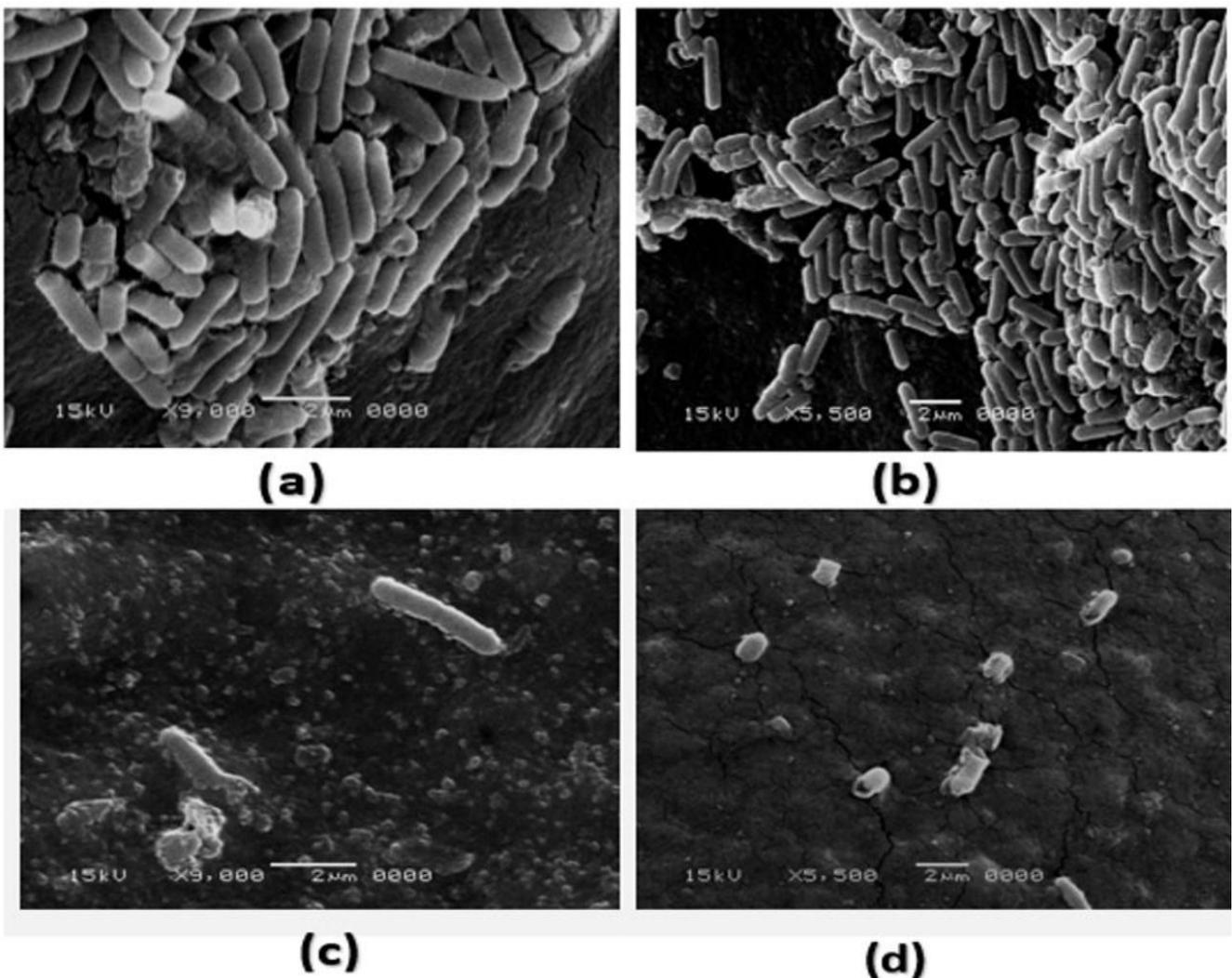


Figure 5. Scanning Electron microscopic pictures of AgNPs treated and untreated *E. Coli* and *Klebsiella* spp. The control cells showed normal, clear and unpenetrated cell membrane (a, b). AgNps treated cells showed cell membrane penetration and perforation of cells leading to excretion of cell metabolites and cell death (c, d)

DISCUSSION

Despite the presence of various modern antimicrobial agents and antibacterial drugs, bacterial infections are still a major threatening problem due to the enormous increase in multi-drug-resistant bacteria. Mobile genetic elements, such as plasmids, are capable of transferring antimicrobial resistance determinant elements among different bacterial populations and play an important role in the epidemiology of antimicrobial resistance (Carattoli, 2013). The extensive misuse of antibiotics has become a leading cause of the emergence of several hazards to public health, such as superbugs, which resist all the current drugs (Khameneh et al., 2016). The clinical effectiveness of beta-lactams has been diminished owing to the massive increase in resistant bacteria and prolonged patient recovery (Denisuik et al., 2013; Mathers et al., 2015). Extended-spectrum beta-lactamases (ESBLs) in pet animals is a threatening issue which has emerged worldwide. Since their emergence, ESBLs have most often been found in *Escherichia coli* and *Klebsiella pneumoniae* (Bonnet 2004; Livermore et al. 2006; Mathers et al. 2015). In the present study, the prevalence of ESBL producing *Escherichia coli* and *Klebsiella* spp. was investigated in different samples obtained from pet animals. MacConkey agar (Oxoid) supplemented by Ampicillin was used for the isolation of ESBL-producing isolates which facilitate screening of the isolates rather than Macconkey supplemented with Cephalosporine as reported by Okapara et al. (2018). ESBL-producing *E. coli* and *Klebsiella* spp. were detected in 23 out of the 120 samples of the current study representing 19.6 % of the total samples. In previous studies, ESBL-producing *K.pneumoniae* were recorded as 41% (Okapara et al., 2018) and 7.5% (Liu et al., 2017). ESBL-producing isolated *E. coli* was detected in 17 (14.16%) samples, 7 from urine, and the other 10 from fecal samples. The obtained results of the current study were indicative of higher percentages, compared to those of ESBL isolated *E. coli* obtained from dogs and cats in Switzerland 8% (Huber et al., 2013) and New Zealand 6.4%, (Karkaba et al., 2019) and lower than those obtained from pet animals in Switzerland 54.7%, (Zogg et al., 2018) and from dairy farms in Germany 75.6%, (Odenthal et al., 2016). Three genes (i.e., *TEM*, *SHV*, and *CTX-M*) are the most predominant in ESBL-producing bacteria (Paterson and Bonomo, 2005). The *blaSHV* and *blaTEM* were detected in all the isolates while *blaCTX-M* was found in 92.3%. Huber et al. (2013) investigated ESBL genes of ESBL producing *E. coli* isolates where *blaCTX-M* was found in 100% of isolates and *blaTEM* in 87.5%. Searching new effective bactericidal alternatives has become an urgent issue for combatting drug resistance. Silver nanoparticles have been established as a promising approach as an alternative for antimicrobial agents in the treatment of several medicinal problems (Beyth et al., 2015; Hassanen and Ragab, 2020). They have attracted great concerted attention and have been broadly used in a variety of applications as antibacterial/antifungal agents in a diverse range of products, including air sanitizer sprays, pillows, respirators, wet wipes, detergents, soaps, shampoos, toothpaste, air filters, coatings of refrigerators, vacuum cleaners, washing machines, food storage containers, cellular phones (Sun et al., 2001). Moreover, they do not cause high level of toxicity in human as well as they have broad-spectrum antibacterial actions (Chandran et al., 2006).

In the present study, AgNPs were tested against ESBL-producing *E. coli* and *Klebsiella* spp. isolates in vitro by the detection of MIC and MBC. Manikprabhu and Lingappa (2014) determined the antibacterial effect of AgNPs with size (28-50nm) against ESBL producing *E. coli* where MIC and MBC were found to be (in the range of 0.11 and 0.22 mg/ml). Moreover, biogenic nanosilver of (20-70nm) was used against ESBL-producing *k. pneumoniae* and *E. coli*, where the recorded MIC and MBC were 1.4µg and 2µg, respectively (Subashini et al., 2014). This indicated that AgNPs had a good bacteriostatic effect according to MIC and good bactericidal effect according to MBC on ESBL-producing *E. coli* and *Klebsiella* spp. As reported, the small size of nanoparticles potentiates the antibacterial effect on microorganisms (Smekalova et al., 2016). Also, AgNPs had the same effect against ESBL- and non ESBL- producing bacteria (Ansari et al., 2014).

It should be noted that it is not easy to compare the obtained results of the antibacterial effect of AgNPs in the current research with those of previous studies since different researchers employed different methods to study the antibacterial effect of AgNPs against different types of bacteria. Besides, the effect of AgNPs against microorganisms was influenced by the size, shape, stability, and concentration of AgNPs (Bandyopadhyay et al., 2018). We found that the concentration of AgNPs used to inhibit or kill microorganisms differed from one another as MIC and MBC values of AgNPs against *E. coli* were lower than those of *Klebsiella* spp.

In the current study, SYBR Green RT-PCR was used to investigate the influence of silver nanoparticles against ESBL-producing *E. coli* and *Klebsiella* spp. resistance genes (*blaCTX-M*, *blaTEM*, and *blaSHV*). The sub-MIC dose (150µg) of silver nanoparticles of average size 58 nm was tested against *E. coli* and showed the downregulation of *blaCTX-M*, *blaTEM*, and *blaSHV* genes with fold change about 0.21, 0.39, 0.53, respectively, for *E. coli*. Furthermore, the sub-MIC dose (310µg) of silver nanoparticles was tested against *Klebsiella* spp. and indicated the downregulation of genes expression with 0.33, 0.65, 0.68 for *blaCTX-M*, *blaTEM*, and *blaSHV* resistance genes, respectively. These results indicated that AgNPs could effectively influence the gene expression of *E. coli* (*blaCTX-M*, *blaTEM*, and *blaSHV*) genes more than that of *Klebsiella* spp, which would subsequently reflect in their resistance pattern.

In the current study, the morphological changes in *E. coli* and *Klebsiella* spp. cells were evaluated before and after the treatment with silver nanoparticles using SEM. The SEM observations in treated cells confirmed cell membrane

damage due to the adherence of AgNPs with bacterial cell membranes and penetration into the cells causing cell death. On the other hand, untreated cells indicated healthy cells with a clear unpenetrated cell membrane. The bactericidal effect of silver nanoparticles is still of unknown mechanism. Many studies suggest that their binding to the bacterial cell membrane may disrupt cell permeability (Kvítek et al., 2008) while other studies propose that the bactericidal effect did not only caused by contact with cell membrane but also because of penetration into the bacterial cell leading to the inactivation of DNA replication and causing cell death (Morones et al., 2005).

CONCLUSION

In the present study, the antibacterial effect of silver nanoparticles was investigated in vitro against Extended Spectrum Beta lactamase producing *E. coli* and *Klebsiella* spp. The findings revealed that using silver nanoparticles as an alternative to antimicrobial agents had an obvious effect on minimum inhibitory concentration, minimum bactericidal concentration, Bacterial cell wall integrity as well as genetically on the expression of antibiotic resistance genes. This trial is very encouraging for the control of antibiotic-resistant bacteria.

DECLARATIONS

Authors' contributions

Omnia A Khalil, Mona I Enbaawy, Eman Ragab, Hossam Mahmoud, and Taher Salah designed the plan of work, supervised the experiment, and revised the manuscript. Eman Ragab is the corresponding author and responsible for English editing, statistical analysis, and formatting the manuscript.

Competing interests

The authors declare no conflicts of interest.

Consent to publish

Written consent was obtained from the owners of the animals after they were informed on the use of their animal samples in the study

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