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*Original article*

# ANTIBACTERIAL EFFECT OF SILVER NANOPARTICLES ON ANTIBIOTIC RESISTANT *E. COLI* O157:H7 ISOLATED FROM SOME DAIRY PRODUCTS

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**Summary**

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Food safety is a worldwide health goal so foodborne diseases are a main health concern. A total 150 of dairy products samples (locally made yoghurt, ice cream and Talaga cheese) (50 for each type) were examined for *E.coli* O157:H7 detection and PCR confirmation using *fliC*$_{H7}$ gene. *E. coli* O157:H7 was detected at 18%, 4%, 8% respectively, in samples. The isolates showed broad antibiotic resistance against vancomycin (84.6%), penicillin G (76.9%), cloxacillin (69.2%) and tetracycline (61.5%). Because of increasing number of microorganisms that are resistant to multiple antibiotics causing continuing economic losses in dairy manufacturing, there is an urgent need for development of alternative, cost-effective, and efficient antimicrobial agents to overcome antimicrobial resistance. Here, silver nanoparticle (AgNPs) solution was prepared, identified by transmission electron microscopy (TEM) with an average size 26.5 nm and examined for bactericidal activity against *E. coli* O157:H7 by using well diffusion assay. The mean inhibition zones of 25 and 50 µg/mL concentrations of Ag-NPs were 15.0±1.2 and 20.9±1.4 mm, respectively. In addition, the statistical analysis showed highly significant differences in the bactericidal effect of different Ag-NPs concentrations on *E. coli* O157:H7 strains. Bacterial sensitivity to nanoparticles is a key factor in manufacture, so nanoparticles were considered suitable for long life application in food packaging and food safety.

**Key words**: antimicrobial agents, cheese, *E. coli* O157:H7, ice cream, locally made yoghurt, nanoparticles, PCR

## INTRODUCTION

*E. coli* O157:H7 belongs to a group of enterohaemorrhagic *E. coli* strains, and is recognised as a pathogen that spreads from food to humans and causes important outbreaks such as haemorrhagic colitis, haemolytic uremic syndrome and thrombotic thrombocytopenic purpura. Toxins produced by the serogroup cause gastroenteritis, bloody diarrhoea and kidney failure which can lead to death in humans. *E. coli* O157:H7 is one of the most significant foodborne pathogenic serogroups

as compared with other *E. coli* strains (Sancak *et al.,* 2015; Wang *et al.,* 2018). *E. coli* O157:H7 serotype is mainly transmitted through faecal contamination of meat, milk and dairy products during the food production process through primary or secondary contact with ruminants' faeces (Myataza *et al.,* 2017).

Many farms use carelessly antimicrobial agents for treatment of animal husbandry diseases. Such agents tend to act directly on the animals' gut causing development of resistance in certain bacteria. Studies from diverse geographical locations in Africa have witnessed global developments of antibiotic resistance among enteric bacteria (Igbinosa, 2016). Multidrug-resistant bacteria have been found in foods for human consumption, streams and effluents and thus represent a hospital, community, and environmental problem (Koga *et al.,* 2015).

Nanotechnology holds a big promise for animal health, veterinary medicine and many areas of animal production. Nanoparticles proved as proficient therapeutic agents due to their outstanding physicochemical properties, characteristics and globally applicable physical mode of action (Meena *et al.,* 2018).

The development of nanosilver products is expanding, they are found in clothing, food containers, wound dressings, ointments, implant coatings, and other items; some nanosilver applications have received approval from the US Food and Drug Administration (Alexander, 2009). Silver nanoparticles (AgNPs) have been intensively studied as antimicrobial agents, including against multidrug-resistant bacteria (Scandorieiro *et al.,* 2016). A positive correlation was reported between the high concentration of AgNPs and the inhibition of *E. coli* isolated from surface and ground water (Dosoky *et al.,* 2015; Meena *et al.,* 2018).

This study was aimed at isolation of multidrug-resistant *E. coli* O157:H7 from some dairy products, evaluation of the antibiotic susceptibility of the isolates and exploring the antibacterial properties of silver nanoparticles against these drug-resistant pathogens.

## MATERIALS AND METHODS

### *Sample collection*

A total of 150 locally made yoghurt, ice cream and Talaga cheese samples (50 for each type), were collected from different localities in Assiut province, Egypt. All samples were collected in sterile separate tubes, labelled and placed in an ice tank to be transferred with a minimum delay to the laboratory for bacteriological examination.

### *Isolation and identification of E. coli O157:H7*

Samples were prepared to isolate *E. coli* O157:H7 as per the standard Bacteriological Analytical Manual (BAM), U.S. Food and Drug Administration (USFDA) method (Sancak *et al.,* 2015). The samples were enriched in modified vancomycin-trypticase soy broth (mvTSB) (Samadpour *et al.,* 1990), a loopful of culture inoculated into Sorbitol MacConkey (SMAC) agar plates. Suspected *E. coli* O157:H7 colonies were sorbitol negative and appeared pale in colour as compared to bright pink sorbitol positive ones (De Boer & Heuvelink, 2000). Various biochemical tests such as sugar fermentation especially sorbitol fermentation test, catalase test, indole production, methyl red, Voges-Proskauer, Simon's citrate agar, urease production, nitrate reduction and

microscopic tests were done for confirmation of *E. coli* O157:H7 as proposed by A.P.H.A. (1992).

*Detection of E.coli O157:H7 isolates using PCR assay*

This part has been done in Molecular Biology Department at Animal Health Research Institute, El-Giza, Egypt authorised by EGAC (ISO 17025/2017) according to Fratamico *et al.* (2000).

*DNA extraction*. DNA extraction from all *E. coli* O157:H7 isolates from previously collected samples were performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications of the manufacturer's recommendations. Briefly, 200 µL of the sample suspension was incubated with 10 µL of proteinase K and 200 µL of lysis buffer at 56 $^{o}$C for 10 min. After incubation, 200 µL of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µL of elution buffer.

*Oligonucleotide primers.* Primers were supplied from Metabion (Germany) to detect *fliC*$_{H7}$ gene of *E. coli* O157:H7. Primers sequences were: GCGCTGTCGA GTTCTATCGAGC and CAACGGTGAC TTTATCGCCATTCC. Amplified segment was 625 bp (Fratamico *et al.,* 2000).

*PCR amplification.* Primers were utilised in a 25-µL reaction containing 12.5 µL of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µL of each primer of 20 pmol concentration, 4.5 µL of water, and 6 µL of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

*Analysis of the PCR products.* The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1× TBE buffer at room temperature using gradients of 5 V/cm. For gel analysis, 20 µL of the products was loaded in each gel slot. Generuler 100 bp ladder (Fermentas, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data analysed through computer software.

*Antimicrobial sensitivity testing of E.coli O157:H7*

The positive PCR isolates were subjected to antibiotic susceptibility testing on Mueller-Hinton agar using the standard disc diffusion method (Adesiyun *et al.,* 2007) against a panel of antibiotics including neomycin S. (30 mg), vancomycin (30 µg), oxacillin (1 µg), novobiocin (30 mg), gentamicin (10 µg), lincomycin (2 µg), cloxacillin (1 µg), penicillin G. (10 U), cephadin (30 µg) and tetracyclin (30 µg) (Difco Laboratories and BioMerieux, France). These antibiotics were selected on the basis of their frequent application in the therapy of *E. coli* related diseases. Briefly, fresh isolates from sorbitol-MacConkey agar plates were cultivated on nutrient agar and incubated at 37 °C for 18–24 h. These fresh colonies were inoculated on sterile physiological saline and standardised to 0.5 McFarland standards. One hundred microliters of the bacterial suspension were spread evenly on the entire surface of Mueller-Hinton agar plates using a sterile swab, after which the antibiotic discs were aseptically placed on the bacterial lawn, and the plates incubated at 37 °C for 18–24 h. At the end of the incubation period, the plates were examined for zones of inhibition and interpreted based on minimal inhibition concentrations (MIC) break-point from the Clinical Laboratory Standards Institute (2014).

*Synthesis of silver nanoparticles*

Silver nitrate ($AgNO_3$) crystal (ACS $AgNO_3$ F.W. 169.87 Gamma laboratory chemicals, assay: 99%), sodium citrate ($C_6H_5Na_3O_7 \times 2H_2O$, purity 99.0%, Sigma-Aldrich) were used. Stable Ag-NPs <100 nm were synthesised by using sodium citrate, the molar ratio of silver nitrate to sodium citrate was 1:7. Silver nitrate was first heated to its boiling point under reflux and then sodium citrate solution was introduced to the reaction. The solution was heated for additional 15 min and cooled to room temperature (Ranoszek-Soliwoda *et al.,* 2017). The solutions were identified and obtained from the Chemistry Department, Faculty of Science, Azhar University, Assiut branch, Egypt. The size of Ag-NPs was measured by transmission electron microscopy (TEM) Model JEOL-JEM-100CX II in the Electron Microscopy Unit, Assiut University, Egypt.

*Bactericidal effect of silver nanoparticles assay*

The isolated *E. coli* O157:H7 strains were used to assess the antibacterial activity of Ag-NPs at concentration 25 and 50 µg/mL by the well diffusion method on Mueller-Hinton agar. The isolates were propagated in BHI broth and incubated at 37 °C for 24 h. The growth density was adjusted to the turbidity of a 0.5 McFarland standard by adding sterile saline to achieve a strain concentration of approximately $1 \times 10^5$ CFU/mL (Gupta *et al.,* 1992) then 0.1 mL of the inoculated broth was streaked into the plates. After 24-hour incubation, the various levels of zones of inhibition were measured (Rajeshkumar & Malarkodi, 2014).

*Statistical analysis*

The prevalence of *E. coli* O157:H7 was calculated and compared by using the Microsoft Excel Spreadsheet. Evaluation of antibacterial effects of different concentrations of silver nanoparticles against *E. coli* O157:H7 isolates were represented as range and means ± standard error and were analysed using paired t-test by Prism software. P values lower than 0.05 were considered significant.

## RESULTS

*E. coli* O157:H7 was recovered from 15 (10%) of the 150 dairy samples examined (Table 1). The organism was detected in 9 (18%) of the 50 samples of locally made yoghurt, 2 (4%) of the 50 ice cream samples and 4 (8%) of 50 Talaga cheese samples.

**Table 1.** Distribution of *E. coli* O157:H7 in some dairy products

| Samples types | Number of samples | *E. coli* O157:H7 | |
|---|---|---|---|
| | | Positive samples number | Positive samples % |
| Locally made yoghurt | 50 | 9 | 18 |
| Ice cream | 50 | 2 | 4 |
| Talaga cheese | 50 | 4 | 8 |
| Total | 150 | 15 | 10 |

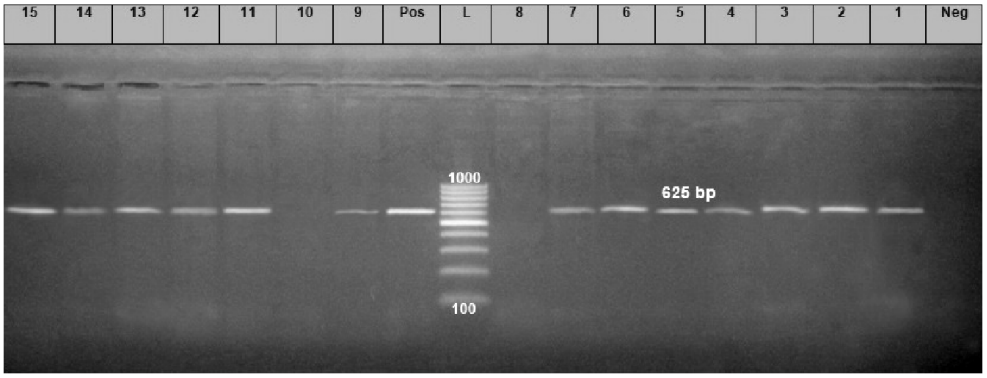


**Fig. 1.** Amplified *E. coli* O157:H7 $fliC_{H7}$ gene recovered from dairy products. L: molecular marker; lane Pos: positive control; lane Neg: negative control; lanes 8, 10: negative isolates (ice cream samples); lanes 1–7, 9, 11–15: positive isolates (locally made yoghurt and Talaga cheese samples).

**Table 2.** Trends on antibiotic susceptibility of 13 positive *E. coli* O157:H7 isolates containing $fliC_{H7}$ gene using PCR. Data are presented as number (%).

| Antimicrobial agents | Percentage profile (n=13) | | |
|---|---|---|---|
| | Resistant | Intermediate | Sensitive |
| Neomycin S | 4 (30.8) | 0 (0) | 9 (69.2) |
| Vancomycin | 11 (84.6) | 2 (15.4) | 0 (0) |
| Oxacillin | 7 (53.8) | 4 (30.8) | 2 (15.4) |
| Novobiocin | 0 (0) | 1 (7.7) | 12 (92.3) |
| Gentamicin | 3 (23.1) | 0 (0) | 10 (76.9) |
| Lincomycin | 2 (15.4) | 3 (23.1) | 8 (61.5) |
| Cloxacillin | 9 (69.2) | 0 (0) | 4 (30.8) |
| Penicillin G | 10 (76.9) | 1 (7.7) | 2 (15.4) |
| Cephadin | 12 (92.3) | 1 (7.7) | 0 (0) |
| Tetracycline | 8 (61.5) | 0 (0) | 5 (38.5) |

All isolated strains subjected to PCR for confirmatory to $fliC_{H7}$ gene by amplified *E. coli* O157:H7 $fliC_{H7}$ gene recovered from dairy products and most of them gave cut at 625 bp except 2 isolates from ice cream samples that gave negative results to used gene (Fig. 1).

All *E. coli* O157:H7 isolates detected in this study were tested for sensitivity to antibiotics. Table 2 revealed that most of them were resistant to vancomycin (84.6%), oxacillin (53.8%), penicillin G (76.9%), cloxacillin (69.2%), cephadin (92.3%) and tetracycline (61.5%). However wide inhibition zones indicated their sensitivity to neomycin S. (69.2%), novobiocin (92.3%) and gentamicin (76.9%).

Synthesised silver nanoparticles were measured by TEM and showed an average size of 26.5 nm and spherical shapes (Fig. 2). When their antibacterial effect at different concentrations (25 and 50 μg/mL) was examined against *E. coli* O157:H7 isolates, significantly different effect was

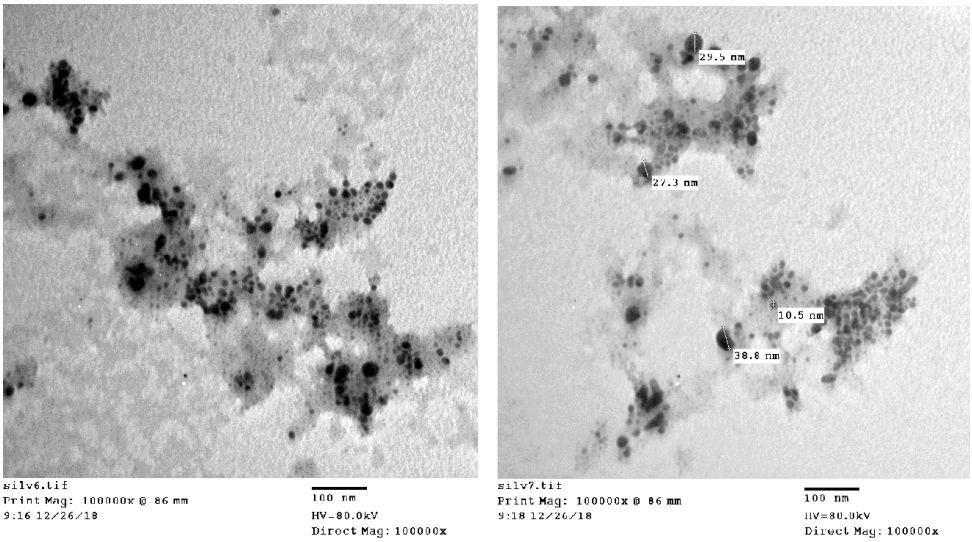


**Fig. 2.** TEM image of spherical Ag-NPs with an average size 26.5 nm.

**Table 3.** Evaluation of antibacterial effects of silver nanoparticles against *E. coli* O157:H7

| Silver nanoparticles concentrations | Inhibition zone (mm) | | | P |
|---|---|---|---|---|
| | Minimum | Maximum | Mean±SEM | |
| 25 µg/mL | 10 | 20 | 15.0±1.2 | 0.0003 |
| 50 µg/mL | 15 | 30 | 20.9±1.4 | |

demonstrated with inhibition zones of 15.0±1.2 and 20.9±1.4 mm respectively for both concentrations (Table 3).

## DISCUSSION

*E. coli* O157:H7 is considered as a major foodborne pathogen transmitted to humans through milk and milk products (Jacob *et al.,* 2013). Therefore, this study investigated the presence of *E. coli* O157:H7 in some dairy products in Assiut city, Egypt and detected the pathogen in locally made yoghurt, ice cream and Talaga cheese with percentages of 18%, 4% and 8%, respectively as shown in Table 1. Presence of such this microorganism in collected samples may be attributed to lack of proper sanitation, use of improper and inexpensive ingredients, bad storage and manufacturing conditions, polluted water. Additionally, it tolerates high acidity and low temperatures; therefore, the pathogen would grow and replicate during storage especially in fermented products as yoghurt (Ateba & Bezuidenhout, 2008; Farrokh *et al.,* 2013; Reuben & Owuna, 2013). Previous studies have detected *E. coli* O157:H7 in nearly similar percentages (7%) in cheese as reported by Oksuz *et al.* (2004) and 3.3% in ice cream by Rahimi *et al.* (2011). Higher precentages were obtained by Metwally & Fatma (2015) (16% and 24%) from Talaga cheese in Beni-Suef and Giza city, Egypt, respectively. Rangel *et al.* (2005) re-

corded that seven outbreaks were associated with dairy products, including 4 from consuming raw milk. The others were due to cheese curds, butter, yoghurt and commercial ice cream bars (possibly due to cross-contamination). Lower rates were recorded by Rahimi *et al.* (2011) who detected *E. coli* O157:H7 in 0 and 4.2% of yoghurt and cheese samples, Sancak *et al.* (2015) and Samuel & Ifeany (2016) having found *E. coli* O157:H7 in 2% of cheese and 8% of yoghurt samples, respectively and Metwally & Fatma (2015) reporting 12% occurrence in locally made yoghurt. Multiple hurdles may be required during processing to provide the required level of protection. As *E.coli* is destroyed by heating, challenges are presented for foods that are consumed raw or after minimum processing (FAO, 2011).

The isolates of *E. coli* O157:H7 in the present study were confirmed by PCR using $fliC_{H7}$ gene which encodes for *E. coli* structural flagellar antigen H7. *E.coli* O157:H7 serotype is most often connected with disease or infection outbreaks (Wang *et al.,* 2002; Myataza *et al.,* 2017). Fig. 1 clarified that all isolates were positive for $fliC_{H7}$ genes except two isolates from ice cream samples. Similar results were recorded by Caro *et al.* (2006) and Rahimi *et al.* (2011) who used $fliC_{H7}$ genes for confirmation of most *E. coli* O157:H7 isolated from dairy products.

*E. coli* O157:H7 isolates showed an increase in susceptibility to different antibiotics, with the highest susceptibility observed to novobiocin, gentamicin and neomycin – 92.3%, 76.9% and 69.2%. respectively. The highest resistance was observed against cephadin – 92.3% followed by vancomycin (84.6%), penicillin G (76.9%), cloxacillin (69.2%) and tetracycline (61.5%). Ateba & Bezuidenhout (2008) carried out an investigation on characteristics of *E. coli* O157:H7 in the North-West Province, South Africa, where all isolates of *E. coli* O157:H7 recovered from cattle were resistant to tetracycline. Rahimi *et al.* (2011) indicated that there was a high resistance of *E. coli* O157 to ampicillin, gentamicin, and erythromycin. Most developing countries such as South Africa tend to use any readily available medication such as antibiotics without first identifying the cause of sickness. This might be one of the factors which have so much contributed to increased bacterial resistance against most antimicrobial agents because resistance in bacteria may be conveyed via bacterial gene transfer (Munita & Arias, 2016). This has led to recently increasing attention towards the use of the silver nanoparticles as antimicrobial agents especially because they are considered nontoxic and environmentally friendly antibacterial materials that may be linked to broad spectrum activity and far lower propensity to induce microbial resistance compared to antibiotics (Dunn & Edwards-Jones, 2004; Eckhardt *et al.*, 2013; Yuan *et al.,* 2017).

The effect of Ag-NPs with an average size of 26.5 nm on isolated *E. coli* O157:H7 at concentrations 25 and 50 μg/mL showed that both concentrations had a bactericidal effect on the pathogen but there was also a significant difference between the two concentrations with respect to bactericidal activity on *E. coli* O157:H7. Our results agreed with Humberto *et al.* (2010) and Paredes *et al.* (2014) who studied the effect of AgNPs at concentrations of 1, 5, 10, and 20–50 μg/mL and found that AgNPs clearly affected the growth curves of *E. coli* O157:H7, especially at 20–50 μg/mL. Indeed, silver nanoparticles attach to the surface of the cell membrane and disturb

its function, penetrate bacteria, and release silver ions (Meena *et al.,* 2018). Lok *et al.* (2007) found that silver nanoparticles target the bacterial membrane, leading to a dissipation of the proton motive force. Consequently, silver nanoparticles need to reach the cell membrane to achieve an antibacterial effect (Quinteros *et al.,* 2016).

The bactericidal activity of silver nanoparticles against multidrug-resistant bacteria could be used in conjunction with advances in impregnation techniques and polymer technology to expand the range of applications of these nanoparticles in the preservation of food, disinfection of medical supplies and equipment, and decontamination of the surfaces of items such as toys and kitchenware (Matsumura *et al.,* 2003; Meena *et al.,* 2018). Especially the size of ~ 1–50 nm and smaller implies the ability to reach structures that otherwise are not available for bigger nanoparticles. Also, at this size, they eliminate bacteria but keep human cells alive because the bacteria have a larger surface area-to-volume ratio than eukaryotic cells, which allows for rapid uptake and intracellular distribution of nutrients and excretion of wastes. This characteristic is achieved by having a rigid cell wall composed of peptidoglycan (Lok *et al.,* 2007; Pal *et al.*, 2007; Ayala-Núñez *et al.*, 2009). For that reason, at the same concentration, silver nanoparticles would be preferentially absorbed and accumulated by bacteria, thus exerting their antibacterial effect without significantly damaging human cells. In addition, silver nanoparticles have been found to bound and disturb bacterial cell membrane activity (Sondi & Salopek-Sondi, 2004).

## CONCLUSIONS

It could be concluded that silver nanoparticles are effective against *E. coli* O157:H7 considered to be an important food pathogen isolated from some dairy products and posing public health hazard. Therefore they could be used as medical care in encompassing setting yet as in silver-incorporating food packaging. So, further investigations were needed for application of silver nanoparticles in food industry as food packaging.

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