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Antimicrobial Activity of Silver Nanoparticles on Pathogenic Bacteria

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Abstract

Nosocomial infection is acquired contamination of hospitals and health care units caused by multidrug resistant bacteria. Currently, bacterial resistance to antimicrobial medication represents a complicated public health problem. Recent studies on the antimicrobial activity of silver nanoparticles (AgNPs) attracted researchers worldwide to focus on the safe synthesis of AgNPs as antimicrobial agents against multidrug resistant bacteria. The antimicrobial efficacy of AgNPs on pathogenic bacteria isolated from clinical cases of acquired hospital infection was targeted in this project. Fifty specimens of stool were collected through private laboratories in Baghdad from patients who suffered diarrheal symptoms. Bacterial isolation, identification, and characterization via culturing on MacConkey agar, Salmonella shigella agar, and IMVic analysis were done besides, using polymerase chain reaction (PCR) through amplifying inf B gene for molecular characterization. The obtained isolates were tested for antimicrobial sensitivity via disk diffusion assay against; Gentamycin, Amoxicillin, Tetracycline, Ceftriaxone and a suspension of silver nanoparticles (1mM AgNo3 reduced by 1% tri-sodium citrate). Results of isolation and IMVic showed the obtained isolates were Klebsiella spp., Enterobacter spp., Citrobacter spp., and PCR assay confirmed their pathogenicity. Disc diffusion assay showed the sensitivity of the isolates (mm); Gentamycin (24.94 ± 0.1), Amoxicillin (2.11 ± 0.13), Tetracycline (12.15 ± 0.1), Ceftriaxone (12.35 ± 0.1). Whereas, all isolates are sensitive to AgNPs (24.12 ± 0.3). This result of the antimicrobial effect of AgNPs on nosocomial infection promises for developing AgNPs solution as a product used in the sterilization of furniture, floors and hospital water cycles.

Keywords: Antimicrobial activity; Nosocomial infection; Pathogenic Bacteria; Resistant bacteria; Silver nanoparticle.

Introduction

Nosocomial infections comprise infections obtained from the hospital or other health care canters, which appear for the first time within 48 hours of admission to the hospital, three days after surgery or 30 days after the surgical operation. Hospital infections had become a major health challenge around the world in the latest decades. Owing to the increase in the number of hospitals and health centres besides emerging of new infections or the re-emerging of past infection and increasing antimicrobial resistance.[1]

Antibiotic resistance still neglecting in developing countries, in part due to the fact that it has not been well documented within regular monitoring systems. Furthermore, uncontrolled and abused uses of antibacterial agents are the main cause to raise the

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resistance against antimicrobial medications and render some of them ineffective after only a few years of their application besides, trade of different types of meat and dairy products[2].

Silver has long been used as an antimicrobial agent in wound healing, both in its solid state and with saline solutions for healthy wounds. Nowadays, dressings saturated with $AgNO_3$[3] can be found, silver shows very interesting properties due to its chemical stability, good conductivity, catalytic activity, and antibacterial. Moreover, AgNPs silver nanoparticles are one of the most widely studied particles at present[4], and AgNPs have been applied in various fields such as textile, cosmetics, food industry and biomedicine. In the field of biomedicine, AgNPs gaining particular strength due to their applications antimicrobial agents, coatings for medical devices, and as carriers for chemotherapy drugs[5]. Although AgNPs have been extensively studied, there is still a need for ongoing research toward developing more biologically sustainable synthesis methodologies, along with uncovering the mechanisms involved in toxicology[6].

Three categories of techniques have been followed in previous research for the preparation of AgNPs[7-8]. Chemical procedures based on the reduction of silver ions in organic or aqueous solution, chemical and physical processes (electrochemical and photophysical) and biosynthesis (by plants). For chemical methods in aqueous solution, various reducing agents have been suggested, such as sodium citrate, sodium borohydride, hydrazine, glucose, ascorbic acid, gallic acid, and various plant extracts[6, 9]. Of the range of chemical methods available for production, chemical reduction is the most commonly used of this type of nanosystem. Chemical synthesis of AgNPs is easy to apply on a large scale and low-priced[4].

AgNPs exhibit effective antibacterial properties against both Gram-positive and Gram-negative bacteria, as well against methicillin-resistant strains[10]. Besides, AgNPs display anti-biofilm activities[11,] and have synergistic action with different types of antibiotics eg, b-lactams, macrolides, and lincosamide[12]. Gram-positive and Gram negative bacteria are significant causes of different infections, principally in hospitals, and are resistant to many antimicrobial agents. Due to the prevalence and rise in bacterial resistance to antibacterial medications, many scientists have been interested in developing antimicrobial agents that are resistance-free and low cost[13]. Such challenges and necessities had led to the use of silver nanoparticles as antiseptics and broad-spectrum activity and far lower susceptibility to induce microbial resistance[14]. Therefore, this study aimed to prepare products of silver nanoparticles; effective against bacterial contamination, eco-friendly, and at low prices for hospital hygiene.

Methodology

Specimen's collection: 50 samples of stool (clinical cases of diarrhoea) were collected from privet laboratories in Baghdad in septic conditions and transported within one hour to the laboratory of bacteriology at Ibn Sina University.

Cultured media and chemicals: all media and biochemical used for the isolation and identification of bacteria were purchased from Hi Media/ India.

Isolation of bacteria: 10g of each stool specimen was inoculated in 90ml flask of sterile peptone water and incubated at 37^{O}C for 24-48hrs.

Then, all inoculated flasks were subjected to test for confirmation of bacterial growth.

Each flask that was positive for bacterial growth was streaked on MacConkey agar (MAC) and Salmonella Shigella agar (SSA) after preparation of serial dilution of bacteria in normal saline[15.]

Identification by IMViC: biochemical identification was done through IMViC test; for each obtained isolate, sterile slants of Indole, Methyl red, Voges-Proskauer and citrate, were stabbed with fresh bacterial inoculum 5x105 then, incubated at 37^{O}C for 24-48 hrs [10].

Molecular detection: was performed through DNA extraction, PCR amplification and gel electrophoresis.

Genomic DNA was obtained according to Wizard genomic DNA purification kit (Promega). A fresh subculture 5x10^{5}cell /ml of fresh inoculum was prepared for each recovered isolates and incubated at 37^{O}C. Isolation of genomic DNA from gram negative bacteria was done according to manufacturer instruction as follow: first, cell pellet was prepared by centrifuging 1ml of culture for 2 min at 13,000 - 16,000 xg, then the supernatant was discarded. A total of 600µl of nuclei lysis solution was added to the cell pellet with gently pipetting to mix well and incubated for 5 min at 80^{O}C then, cooled to room temperature. About 3 µl of RNase solution was added mixed and incubated for 15-60 min at 37^{O}C

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then, cooled to room temperature. A total of 200µl of protein precipitation solution was added and vortex for cell precipitation and the sample was incubated on ice for 5min. After that the sample was centrifuged for 3 min at 13,000-16,000 xg. About 800µl of supernatant was transferred to a clean tube containing 600µl of 99% Isopropanol at room temperature with inverting the tube until the white thread of DNA appeared. The samples were centrifuged for 2 min at 13,000-16,000 xg and the supernatant was discarded for DNA precipitation and rehydration. About 600µl of 70 % ethanol was added and mixed by upside down inverting then, centrifuged for 2 min at 13,000 -16,000 xg. Ethanol was aspirated and the pellet was air dried for 10 -15 min. A total of 100µl of rehydration solution was added to rehydrate the DNA pellet for 1 h at 65 ^{O}C or the pellet could be kept overnight at 4^{O}C.Then, DNA was electrophoresed on 7% agarose gel and detected by UV gel doc device. All positive samples of DNA are stored at -20^{O}C until use.

DNA amplification: All reactions were prepared for all isolates in a final volume 25µl/0.5ml PCR tube. Each reaction was contained (Master Mix 12.5µl, F primer-1.25µl, R primer-1.25µl, free RNA water 5µl and DNA template 5µl in addition to control). following the PCR protocol: Initial denaturation starting at 95°C for 5mim then, 1min. Annealing temperate ranging 68°C for 90sec. Initial extension at 72°C for 90s followed by final extension for 5min. The PCR products were seen by UV after electrophoresing on 1.5% agarose gel/100volt. Both forward and reverse primers, Table.1 were designed by[16]

Chemical synthesis of nanoparticles: Synthesis of silver nanoparticles was done under constant heating through reducing $AgNo_3$ (starting agent) by tri-sodium citrate $C_6H_5O_7Na_3$. 17.0mg of silver nitrate was added to 250 ml deionised water to prepare 1mM of $AgNO_3$ solution and adjusted in a microwave oven for constant heating to let the solution dissolves absolutely. 10 ml (1%) solution of tri-sodium citrate was added to $AgNO_3$ solution drop by drop with mixing vigorously. The mixture was heated and the colourless solution changed to yellowish brown. The solution was the removed from microwave oven and still at room temperature with stirring to cool at 28^{O}C[17].

Antimicrobial sensitivity: to evaluate the efficacy of $AgNO_3$ solution as an antimicrobial agent; discs were prepared from filter paper and autoclaved, then dispense in the $AgNO_3$ solution until soaked all the solution and dried. At the same time antibiotic sensitivity test was performed in the disc diffusion technique. Moller Hinton plates were prepared and streaked with 0.5 MacFerland of a fresh culture of each isolate/double.

Then, the plates were dispensed with antibiotic discs; Gentamycin, Amoxicillin, Tetracycline and Ceftriaxone. Besides AgNPs dried filter pares were dispensed on Moller Hinton plates also streaked with 0.5 MacFerland of a fresh culture of each isolate/double.

Results and Discussion

Results

Isolation outcomes showed; out of 50 stool cultured specimens, 26 isolates were positive to grow on MacConkey agar (the differential media to lactose ferment bacteria of *Enterobacteriaceae*), and pink colonies due to lactose ferment were seen. Whereas, none of the isolates were positive to grow on SSA with gas production, Table.2. Gram stained smears of collected isolates were viewed by light microscopic; short rods, red staining and no spore forming.

Polymerase chain reaction PCR assay revealed that all 26/50 of lactose ferment isolates were positive to detection by PCR-*inf* gene with an expected size of 647bp, Fig 1.

Table 1. *inf* B primers designed for DNA amplification by PCR.

Primer	Nucleotides sequence	Reference
Forward *inf* B	5'ATYATGGGHCAYGTHGAYCAYGGHAARAC'3	**Hedegaard *et al.*,(1999). (15)**
Reverse *inf* B	5'TATCCGACGCCGAATCCGRTTNCGCATNG CNCGNAYNCGNCC'3	**Hedegaard *et al.*,(1999). (15)**

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Table 2. Results of IMViC analysis and PCR assay for characterization and identification of collected isolates.

Collected isolates	IMViC analysis				Total	Contaminate specimen frequency %	PCR Reaction
	Indole	Methyl red	Voges-Proskauer	Citrate	26		
***Citrobacter* spp.**	-	+	-	+	8	30.8	+
***Enterobacter* spp.**	-	-	+	+	9	34.6	+
***Klebsiella* spp.**	-	-	+	+	9	34.6	+

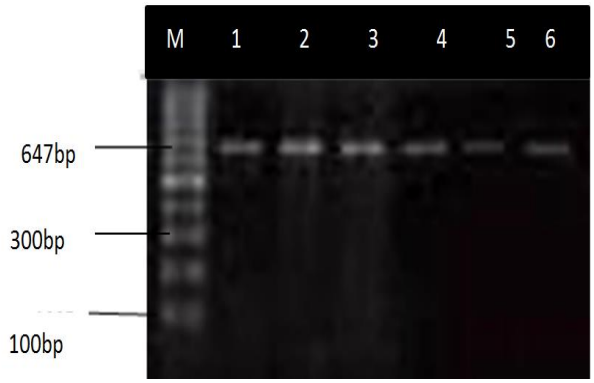

Figure 1. PCR products for amplification of *inf* B gene (647bp).
Lane1: DNA marker 1100bp, lane1-5 collected isolates,
lane6: control (*E. coli* isolate characterized by api 20 system).

Chemical synthesis of silver nanoparticles showed the dark brown colouration of reduced solution of $AgNO_3$ after continuous heating for 15min and above.

Antimicrobial activity of the cooled AgNPs displayed strong and constant ability as an antimicrobial agent against all collected isolates, AgNPs (24.12 ± 0.3). Whereas; the antibiotic discs showed different ranges of antimicrobial efficacy against the same isolates; Gentamycin (24.94 ± 0.1), Amoxicillin (2.11 ± 0.13), Tetracycline (12.15 ± 0.1), Ceftriaxone (12.35 ± 0.1), Table.3.

Table3. Results of antimicrobial sensitivity assay and antimicrobial activity of silver nano particles on clinical bacterial isolates.

Collected isolates	Antimicrobial sensitivity/Disc diffusion assay (mm)				AgNPs Antimicrobial activity
	Gentamycin	Amoxicillin	Tetracycline	Ceftriaxone	
Citrobacter spp.	24.97[a]	2.10*	12.13[b]	12.34[b]	24.11[a]
***Enterobacter* spp.**	24.92[a]	2.10*	12.15[b]	12.37[b]	24.13[a]
***Klebsiella* spp.**	24.90[a]	2.12*	12.16[b]	12.34[b]	24.14[a]
Total	24.94 ± 0.1	2.11 ± 0.13	12.15 ± 0.1	12.35 ± 0.1	24.12 ± 0.3

a,b Statistical significant difference (P<0.05), a>b *Non-significant.

Discussion

Results of this study showed the antimicrobial efficacy of AgNPs against pathogenic bacteria of nosocomial infection. Globally, health care associated infections have been grown into a public health concern[-18]. The nosocomial infection ranges in developed and developing countries between 7 to10%. This variation in antimicrobial sensitivity refers to many factors such as; the prevalence of resistant pathogens, ill-use of antibiotics, the severity of the infection and a long stay in hospital. It's very necessary to control nosocomial infection since it becomes one of the death cause among hospital staying patients in recent years[3]. Outcomes of this study revealed the

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prevalence of gram-negative, lactose ferment, bacteria 52% among pathogens associated with hospital infection is too high, and agreed with the results of Tolera M et al,[19]. Besides, results of this study reported *Citrobacter* spp., *Enterobacter* spp. and *Klebsiella* spp., concordant with outcomes obtained by[19,20]. The three major sites for nosocomial infection include the urinary tract system (31%), the respiratory system (24%), and the bloodstream (16%). Nosocomial infection can also occur in the skin and other organs. Pneumonia, urinary tract infection, and septicaemia are the most commonly diagnosed nosocomial infection on the three major sites. It has been reported that Ventilator-associated Pneumonia (VAP) is the most common nosocomial infection in the intensive care unit (ICU) and responsible for approximately fifty percent of all hospital-acquired pneumonia cases and UTI is the most common NIs in the developed countries.[21]

Chemical mediated synthesis SNPs and their efficacy as antibacterial against Gram negative bacteria were described by[1]. They proposed that SNPs penetrate cell wall of G- bacteria and attach to the cell membrane at surface and releasing Ag ions that disturb its function as well [9, 21] were suggested the same to antibacterial activity against gram positive bacteria[22,23].

Conclusion

The present study is principally concerned with chemically mediated synthesis of silver nanoparticles through reducing of silver with trisodium citrate and obtained silver nanoparticles. Besides, the efficacy of SNPs on G- bacteria isolated from clinical cases of hospital acquired infection was approved. Therefore, the promising results encourage to the development of SNPs products to use in the hygiene of hospitals. Furthermore, studies on the efficacy of SNPs on G+ bacteria are required to enhance the need for SNPs products as detergents.

Author's Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Authors sign on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee in the Iraqi Ministry of Science and Technology.

Author's Contribution Statement

Gh. AL.: proposed the project, and participated in bacteriological analysis and writing. M. A. A.: participated in sample collection and analysis of antibacterial activity. S. H. K.: participated in literature reviews and preparation of nanosilver particles and related materials. All authors funded the project and publication fees.

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النشاط المضاد للميكروبات لجسيمات الفضة النانوية على البكتيريا المسببة لألمراض المعزولة من الحاالت السريرية لعدوى المستشفيات

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الخلاصة

عدوى المستشفيات هي تلوث المستشفيات ووحدات الرعاية الصحية الناتجة عن البكتيريا المقاومة لألدوية المتعددة. في الوقت الحالي ، تمثل مقاومة البكتيريا لألدوية المضادة للميكروبات مشكلة صحية عامة معقدة, اجتذبت الدراسات الحديثة الباحثين في جميع أنحاء العالم حول النشاط المضاد للميكروبات لجسيمات الفضة النانوية(AgNPs(للتركيز على التوليف االمن لـAgNPs كمضادات للبكتيريا المقاومة لألدوية المتعددة. تم استهداف الفعالية المضادة للميكروبات لـ AgNPs كعوامل مضادة للبكتريا المسببة لألمراض المعزولة من الحاالت السريرية المكتسبة من عدوى المستشفى في هذا البحث. تم جمع خمسين عينة من البراز من المختبرات الخاصة في بغداد من مرضى يعانون من أعراض اإلسهال. كما تم إجراء العزل والتعرف والتوصيف البكتيري عن طريق الزراعة على أجار MacConkey و Salmonella agar shigella وتحليل IMVic باإلضافة إلى استخدام جين RNA-S16 PCR للتوصيف الجزيئي. اختبرت العزالت التي تم الحصول عليها من حيث الحساسية للمضادات الميكروبية عن طريق مقايسة االنتشار القرصي ضد؛ جنتاميسين ، أموكسيسيلين ، تتراسيكلين ، سيفترياكسون ومعلق من جزيئات الفضة النانوية)1 ملي موالر 3 AgNoمخفف بنسبة ٪1 سترات ثالثية الصوديوم(. أظهرت نتائج العزل IMVicو PCR أن العزالت التي تم الحصول عليها هي .spp Klebsiella و .spp Enterobacter و spp Citrobacter.، والتي أكدت قدرتها المرضية. أظهر فحص انتشار القرص حساسية العزالت)مم(؛ جنتاميسين)24.94 ± 0.1(، أموكسيسيلين)2.11 ± 0.13)، تتراسيكلين)12.15 ± 0.1(، سيفترياكسون)12.35 ± 0.1(. حيث أن جميع العزالت حساسة لـ AgNPs(24.12 ± 0.3).هذه النتيجة للتأثير المضاد للميكروبات لـ AgNPs على عدوى المستشفيات يعد بتطوير محلول AgNPs كمنتج يستخدم في تعقيم أألثاث واألرضيات والمرافق الصحية المستشفى

الكلمات المفتاحية: نشاط مضادات الميكروبات ؛ عدوى المستشفيات ؛البكتيريا المسببة للأمراض؛ البكتريا المقاومة ؛ جسيمات الفضة النانوية.