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Multidrug resistance and phylogenetic analyses of *Pseudomonas aeruginosa* based on the *16S rRNA* gene of isolates recovered from clinical samples and their susceptibility to silver-nanoparticle

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ABSTRACT

Background: *Pseudomonas aeruginosa* is a highly antimicrobial-resistant pathogen with a very narrow range of effective antibacterial agents. Therefore, finding alternative compounds is highly required, such as silver nanoparticles (AgNPs).

Aim: The current study was conducted to identify the multidrug resistance (MDR) profile and perform a phylogenetic analysis on *P. aeruginosa* isolates recovered from clinical samples (human, cows, cats, and fish) and to study their susceptibility to AgNPs.

Methods: 40 samples were subjected to conventional cultivation and biochemical analyses to identify *P. aeruginosa*. Moreover, these isolates were tested for their antibiotic resistance profile and their response to AgNPs using disk diffusion methods. PCR and Sanger-based sequencing were performed using the 16S rRNA gene as a target.

Results: The results showed that all isolates were resistant to cefixime and sensitive to meropenem. Conversely, the AgNPs were effective in producing larger zones of inhibition. The PCR revealed amplification of the target, and the sequencing and phylogenetic tree of four isolates revealed close similarity with global human sequences from different regions.

Conclusion: The study reveals the MDR characteristics of *P. aeruginosa*. The isolates are highly susceptible to silver nanoparticles.

Keywords: Alternative medicine, Antibiotic resistance, Multidrug resistance, Nosocomial infection, *Pseudomonas aeruginosa*.

Introduction

Pseudomonas aeruginosa is a biofilm-producing pathogen, and one of the most important multidrug-resistant (MDR) pathogens involved in hospital infections (Pang *et al*., 2019). *P. aeruginosa* infections are life-threatening to patients particularly those in intensive-care units and/or those with burns or wounds and/or post-endoscopy.

The most urgent problems are initiated by resistant medical strains collectively called "ESKAPE" (Huang *et al*., 2011). ESKAPE is an acronym for the Enterococcus faecium, *Staphylococcus aureus*, and the three four Gram-negative bacteria, such as *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and Enterobacter spp. Most of these isolates are MDR and are the greatest contributors to nosocomial infections worldwide. The clinical importance of the ESKAPE pathogens is related to their frequent hypervirulence, and the presence of several mechanisms of antibiotic resistance that directly explain failures of therapy, prolonged antimicrobial treatments, and increased treatment costs (Rice, 2008; Rice, 2010).

Nanotechnology or nanoparticles (NPs) loaded with antimicrobial substances may also be used to replace antibiotics using a size between 10 and 100 nm. NP-based antimicrobial agents can directly kill specific bacteria and deliver additional antibacterial substances. According to that, NPs should be very effective against

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MDR strains. NPs can directly target and damage a pathogen cell wall without the need to enter it. NPs are multifaceted in terms of the elimination of both Gram-positive and Gram-negative bacteria (Mohanraj and Chen, 2006; Michiels *et al*., 2016; Wang *et al*., 2017; Gupta *et al*., 2019).

One of the advancements in the field of nanoscience is the use of silver nanoparticles (AgNPs). AgNPs are among the most extensively studied NPs due to their unique properties and numerous applications. Notably, their bactericidal efficacy has shown great promise as an option against antibiotic-resistant bacteria (Ramalingam *et al*., 2016). The biosynthesis of AgNPs has recently gained a lot of importance and attention from scientists worldwide. Their wide applicability stems from their low cost, rapid synthesis, shape and size-controlled synthesis, high stability, effectiveness, biocompatibility, and safety (Tang and Zheng, 2018).

The current study was conducted to identify the MDR profile and perform a phylogenetic analysis on *P. aeruginosa* isolates recovered from clinical samples (human, cows, cats, and fish) and to study their susceptibility to AgNPs.

Materials and Methods

Collection of clinical samples

Forty clinical samples were collected from humans, cows, cats, and fish, from February to May, 2023 in Al-Diwaniyah Teaching Hospital and Private veterinary clinics, Al-Diwaniyah Province, Iraq (Table 1). The samples were collected and delivered right away to the laboratories of College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq, for bacterial culturing and molecular techniques.

Isolation of P. aeruginosa strains

After transporting to the laboratory, the swabs were exposed to cultivation on MacConkey agar, blood agar, and nutrient agar and incubated at 37°C for 24 hours. After incubation, subculture on selective *Pseudomonas* Cetrimide agar and *Pseudomonas* Chromogenic agar for selective growth. All culture media were prepared following company guidelines.

Antimicrobial susceptibility

The test was performed using the AST with the Kirby Bauer Method (Mishra *et al*., 2021), which is an agar disk diffusion method. Table 2 is the list of antibiotic discs and their quantity of antibiotics used against *Pseudomonas* spp. The test was prepared with 0.5 dilution of bacterial culture inoculated onto a Mueller-Hinton agar plate by spreading with a sterile cotton swab (Barry and Thornsberry, 1985; Bauer *et al*., 1966). After the plates were left undisturbed for 15 minutes, the antibiotic discs were placed onto the plate, and the incubation was done at 37°C for 24 hours according to the standards of the Clinical Laboratory Standards Institute (CLSI). The diameter of the inhibition zone was checked to confirm the bacterial susceptibility to the antibiotics, and the result was interpreted by the given numerical values from CLSI (Poletto and Reis, 2005; Clinical and Laboratory Standards Institute, 2006).

Table 1. Source of samples and site of collection.

Sample source	Sample amount	Type of infection
Human	10	Wound burns
Cow	10	Mastitis
Cat	10	Contamination wound after ovariectomies
Fish	10	Gill necrosis

Antimicrobial activity of AgNPs

The synthesis of AgNPs was conducted by chemical reduction and precipitation methods as illustrated by Khalil *et al* (2017). The disc diffusion on solid Mueller Hinton agar was used (Saviuc *et al*., 2011). The bacterial strains were adjusted to the density identical to the 0.5-McFarland scale.

The nanometer-scale silver particles used in the study were applied via the disc diffusion technique, using exposures in 5 µl amounts of each stock solution of the tested product, including various concentrations (12.5, 25, 50, and 100 µg/ml), in addition to the control (AgNPs only), which was also at a concentration of 12.5 µg/ml. Nuclease-free water was tested as an analogue for comparison, as a solvent base in order to have toxicity effects. Incubation was done at 37°C and assessing the results after 24 hours by measuring the inhibition zones of bacterial growth.

Genomic DNA extraction

Purified genomic DNA from MDR *P. aeruginosa* was isolated using the Presto™ Mini gDNA Bacteria Kit, referred to as precellys® premium genomic kit, according to the manufacturer's instructions. Then purified DNA was preserved at −20°C until PCR processing.

Primer design

The *Pseudomonas* genus was identified by using specific primers of 16S rRNA gene sequences available in the GenBank database designed using Primer3 and checked for validity using NCBI Primer-BLAST (Table 3).

Molecular detection of P. aeruginosa

For confirmative molecular detection, the samples were subjected to PCR assays. The PCR was carried out in a 20 µl reaction mix consisted of 8 µl Taq DNA polymerase, 1 µl (10 pmol) of each primer, 5 ml of DNA template, and 5 µl PCR water. The following program was used as initial denaturation at 95°C for 5 minutes and then denaturation at 94°C for 30 second, annealing at 60°C for 40 seconds, and elongation at 72°C for 30 seconds which was followed by final extension at 72°C for 5 minutes (Thermo cycler; Eppendorf, Germany). The amplified products were revealed by UV light, after determined by running in the 1% agarose

Table 2. Discs of Antibiotics and their amount used.

Antibiotics disc	Gentamicin (CN)	Amikacin (AK)	Meropenem (MPM)	Cefixime (CFM)	Ciprofloxacin (CIP)
Amount	10 µg	10 µg	10 µg	5 µg	10 µg

Table 3. Primers of *P. aeruginosa*.

Primer	Sequence	Amplification
16s rRNA	Forward: TCAACCTGGGAACTGCATCC	668 base pair
	Reverse: CAGACTGCGATCCGGACTAC	

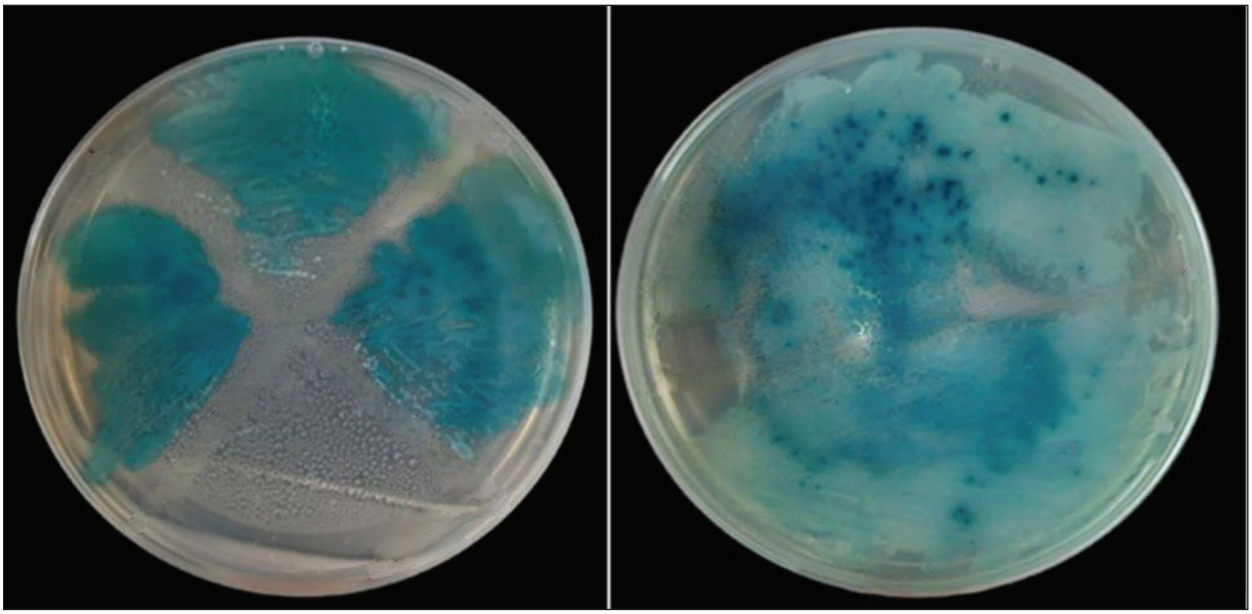

Fig. 1. *Pseudomonas aeruginosa* on chromogenic agar from clinical samples (human, cows, cats, and fish).

gel containing ethidium bromide. Positive control was used of known bacterial DNA, and negative control of no DNA was used from the current study isolates.

Identification of P. aeruginosa using 16S rRNA gene sequencing analysis

The DNA sequencing has achieved the identification of *P. aeruginosa* based on the sequencing of the purified PCR (used as a confirmative test for the presence of the 16S rRNA gene) products of four samples, one from each group, purified from the agarose gel using an EZ EZ-10 Spin Column DNA Gel Extraction Kit from Biobasic, Canada and was sent to Bioneer Company in Korea. The DNA sequencing was performed using an AB DNA sequencing system to study the phylogenetic statuses of the bacterium. The genomic sequences were obtained and uploaded to GenBank-NCBI for public access. A process of multiple sequence alignment was performed using the Basic Local Alignment Search Tool. The phylogenetic tree was created, and phylogenetic analysis was performed using the MEGA-X program to draw the phylogenetic tree.

Ethical approval

Not needed for this study.

Results

Identification of P. aeruginosa

Following a 24-hour incubation period at 37ºC, *P. aeruginosa* was presumed due to their colonies on cetrimide agar (the greenish–blue pyocyanin-colored colonies) and chromogenic agar. The colonies are shown in Figures 1 and 2.

Antibiotic and AgNPs sensitivity and susceptibility

Table 4 and Figure 3 show the four group samples that were evaluated against 5 antibiotics. All isolates exhibited resistance to cefixime and were intermediately sensitive to amikacin while all isolates showed high sensitivity to meropenem and gentamicin. In addition, MDR was seen in four classes of antibiotics,

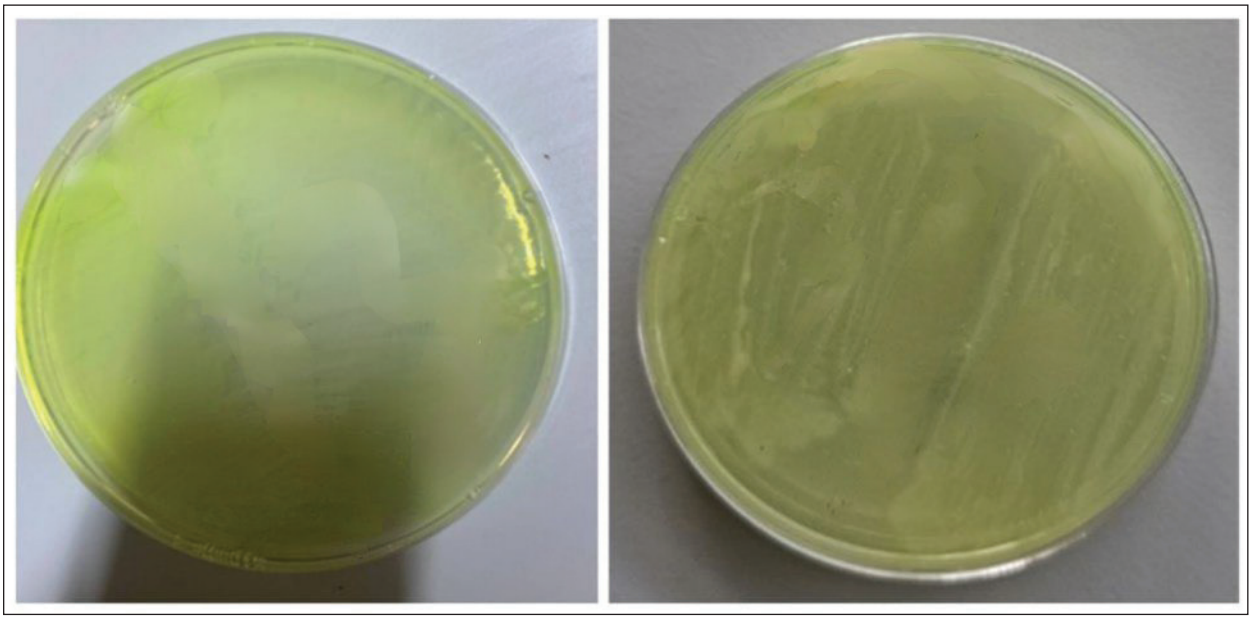

Fig. 2. *Pseudomonas aeruginosa* growth on cetrimide agar from clinical samples (human, cows, cats, and fish).

Table 4. Mean of inhibition zone diameter of antibiotics (amikacin (AK), cefixime (CFM), ciprofloxacin (CIP), gentamicin (CN), and meropenem (MPM)) against *P. aeruginosa* isolated from clinical samples (human, cows, cats, and fish).

Antibiotic disc	AK 10 µg	CFM 5 µg	CIP 10 µg	CN 10 µg	MPM 10 µg
Zone of inhibition(mm)	8.46 ± 4.23	0 ± 0	9.53 ± 4.76	11.47 ± 5.73	11.69 ± 5.84

which included aminoglycoside, carbapenems, fluoroquinolones, and cephalosporin. The inhibition zones of AgNPs against *P. aeruginosa* were at 19.75 ± 2.25 mm at the concentration of 100 µg/ml.

Furthermore, the larger inhibition zone was produced at the highest concentration of AgNPs. For instance, the concentration of 100 µg/ml had a wider inhibition zone as compared to the concentration of 50, 25, and 12.5 µg/ml, as exhibited in Table 5, Figures 3 and 4.

Molecular detection of P. aeruginosa

The results of PCR identification by a species-specific primer of *P. aeruginosa* showed positive amplification only in 15/40 samples. Table 6 illustrates the results of employing the 16s rRNA gene to diagnose *P. aeruginosa* with a product size of 668 bp (Fig. 5). The amplified products were visualized on a 1% agarose gel stained with ethidium bromide and viewed under UV light. The presence of a 668 bp band indicated a positive result for *P. aeruginosa*

16S rRNA sequencing

The 16s rRNA gene was sequenced (accession numbers: PP514,724.1, PP514,725.1, PP514,726.1, and PP514,727.1) to construct a phylogenetic tree. The sequence of these samples was compared with global reference strains. The constructed phylogenetic tree by the MEGA-X software showed that all the local strains were found to be completely identical with isolates obtained from human samples and highly related strains of *P. aeruginosa* (Table 7; Fig. 6).

Discussion

Pseudomonas aeruginosa can acquire and keep a lot of resistance determinants (18). Because of the intrinsic and acquired resistance to antimicrobial agents, few categories of antibacterial agents are still effective to treat *P. aeruginosa* infections. Thus, MDR outbreaks of *P. aeruginosa* infections are still observed in hospital worldwide despite effective infection control interventions (Hirsch and Tam, 2010; Singh *et al*., 2012).

All clinical samples showed 100% sensitivity to cefixime. Meropenem was the best of the antibiotics in our test. The outcomes of our study disagree with the work of Bhuiya and Sarker in 2018 who reported that all isolates were resistant to cefixime and highly sensitive to meropenem (Hong *et al*., 2015). *Pseudomonas aeruginosa*, the bacteria responsible for human infection and contamination of bio and healthcare environment, has greater resistance to β-lactam antibiotics than non-β-lactam antibiotics this is due to the mass production of β-lactamase because of the resistance gene and the mutational processes (Janam *et al*., 2011; Mahmudullah *et al*., 2018). Our study shows that amikacin has a mild effect on *P. aeruginosa*, our finding is different from

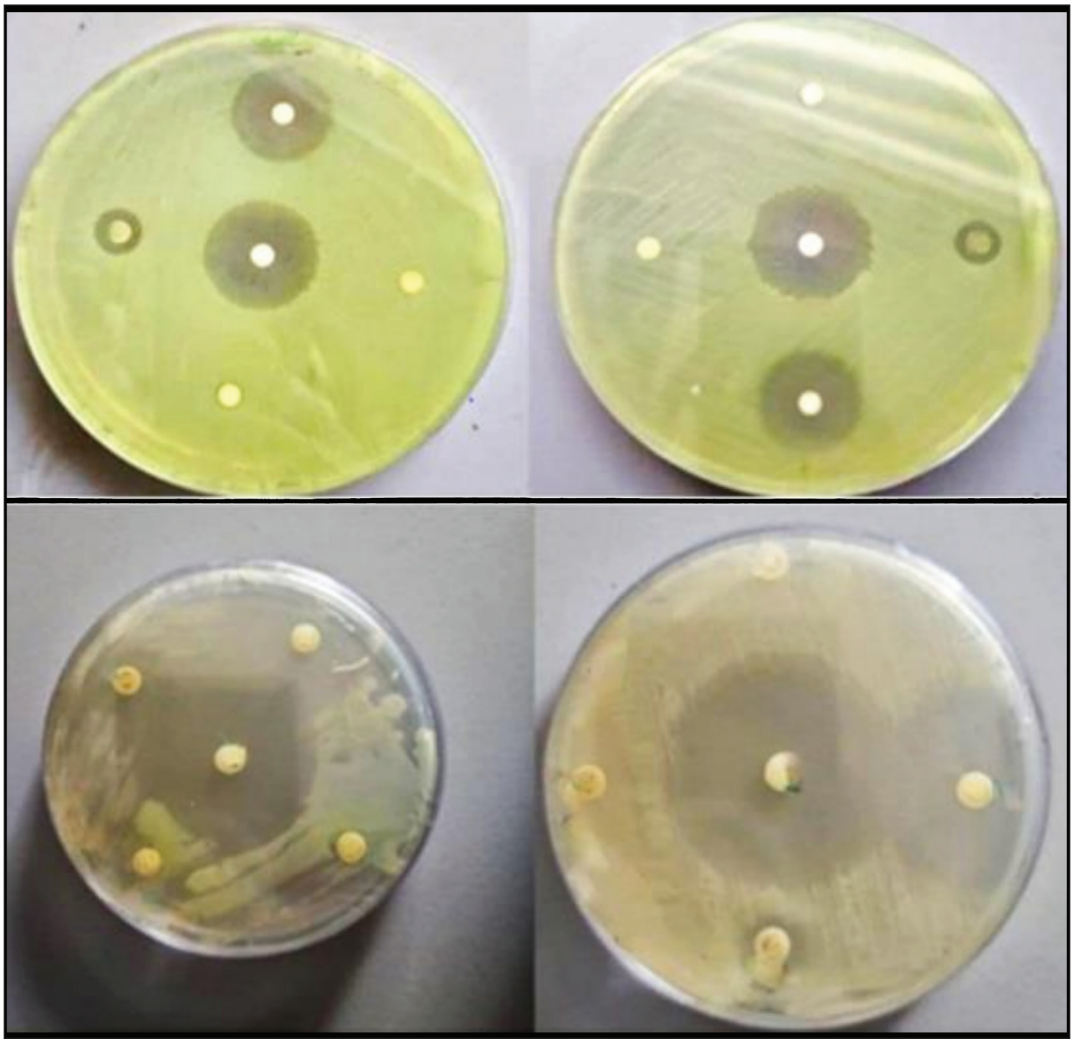

Fig. 3. Antimicrobial activity of AgNPs against four isolated groups of *P. aeruginosa* from clinical samples (human, cows, cats, and fish). Cefixime, amikacin, meropenem, and gentamicin were used as controls.

Table 5. Mean of inhibition zone diameter of sliver NPs against *P. aeruginosa* isolated from clinical samples (human, cows, cats, and fish).

Nano concentration	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
Zone of inhibition(mm)	2.75 ± 1.10	9.25 ± 1.65	18 ± 1.22	19.75 ± 2.25

the high sensitivity of *P. aeruginosa* to amikacin which was reported by the work of Javiya *et al* (2008).

Amikacin was developed to be selective as a poor substrate to the β-lactamases through phosphorylation, adenylation, or acetylation. However, some organisms develop enzymes that can still deactivate the agent. Amikacin can be a good treatment for pseudomonal infections, and it should be used only for serious hospital infections to avoid a rapid selection of resistant strains. We observed in our experiment that high concentrations of AgNPs showed major inhibitory

Table 6. PCR results and subsequent sequencing of *P. aeruginosa* isolated from clinical samples (human, cows, cats, and fish). $N = 40$.

Samples	Positive-PCR	Sequencing
Human-10	3	1
Cattle-10	4	1
Cat-10	4	1
Fish-10	4	1

zones around *P. aeruginosa*. Our finding is consistent with the work of Javiya *et al* in 2008 and Salih in 2017 (Salih *et al*., 2017; Aldujaily *et al*., 2023). The antibacterial effect of silver ions is due to their positive charges which attract the negatively charged microbe via electrostatic interaction. The net attraction is expected to be more important whatever of factors that affect the losses of bacterial cells such as their size and shape (Przemieniecki *et al*., 2022).

AgNP continuously releases silver ions after dissolution, which is a crucial feature of silver NPs. The affinity between silver ions to negatively charged proteins and nuclei is responsible for their effect on the mitigation of the bacterial cell wall, membrane, and nucleic acid function. Direct exposure to AgNP always leads to partial or total damage to the cell membrane and produces reactive oxygen species, which can then lead to the production of bactericidal free radicals (Wu *et al*., 2014).

The 16S rRNA gene is present in almost all bacterial species except for Mycoplasma species. Sequence variation in the target bacterium was quantified using typically one or two long variable-length 16S rRNA primers that cover a broad phylogenetic range of most organisms. The more conserved things are the greater the stringency. Variation will be greater in variable regions of the 16S rRNA regions. The sequence pattern identified was then used for the identification of the unknown species. Previous data suggest that 16S rRNA can identify a genus in 65%–90% of cases (Janda and Abbott, 2007; Woo *et al*., 2008; Akram *et al*., 2017).

In the GenBank Database, the global strains of humans, cows, cats, and fish are different from the current strains in Iraq. Second, the tree shows that the strains in the current study were 100% similar to each other, and most genetically similar to strains from China. The clear tree branching for all isolates showed powerful diversity of the bacterium within the genus.

The study reveals the MDR characteristics of *P. aeruginosa* isolated from clinical samples (humans, cows, cats, and fish). The isolates are highly susceptible to silver NPs.

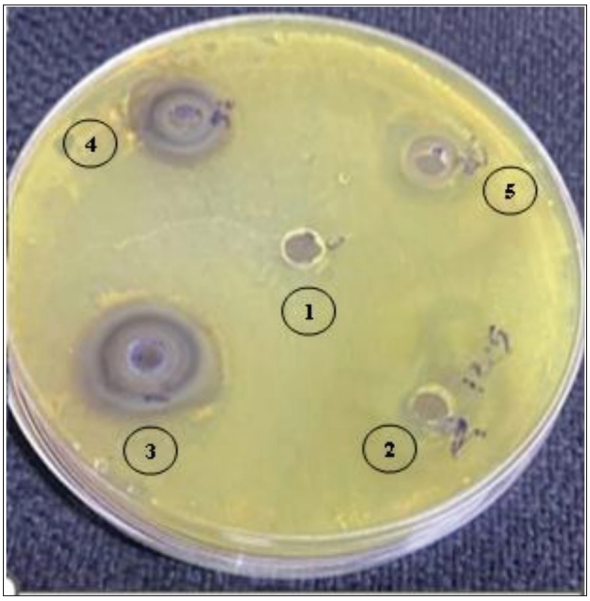

Fig. 4. Antibacterial effects of AgNPs against *P. aeruginosa* isolated from clinical samples (human, cows, cats, and fish). 1. Control (Negative), 2 100 µg/ml AgNPs, 3. 50 µg/ml AgNPs, 4. 25 µg/ml AgNPs, and 5. 12.5 µg/ml AgNPs.

Table 7. Current study *P. aeruginosa* isolates and their nucleotide-based similar global isolates.

Accession No.	Country	Identity this study
PP514724.1	Iraq	100%
PP514725.1	Iraq	100%
PP514726.1	Iraq	100%
PP514727.1	Iraq	100%
OL314556.1	Iraq	100%
MK240441.1	china	99.83
MH769236.1	china	99.83

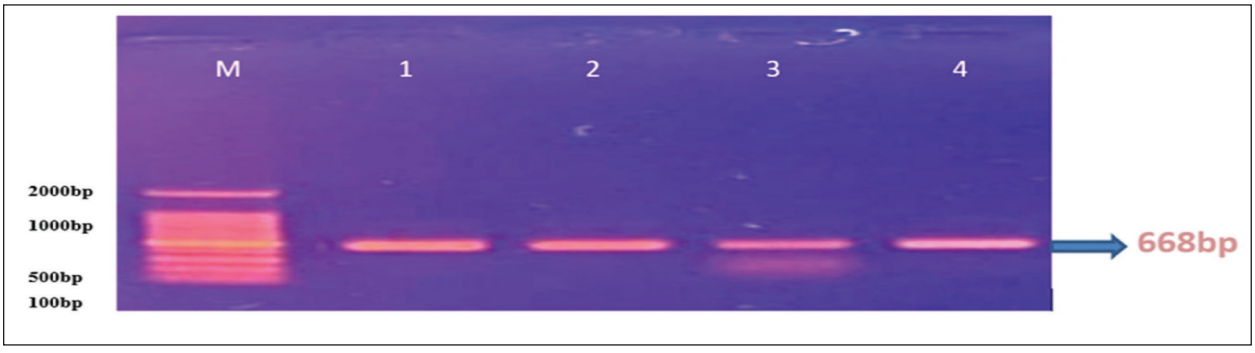

Fig. 5. Agarose gel electrophoresis for amplified (668 bp) *P. aeruginosa* specific 16S rRNA gene isolated from clinical samples (human, cows, cats, and fish). M: DNA ladder = 100 bp. Lanes 1–4: Positive amplification.

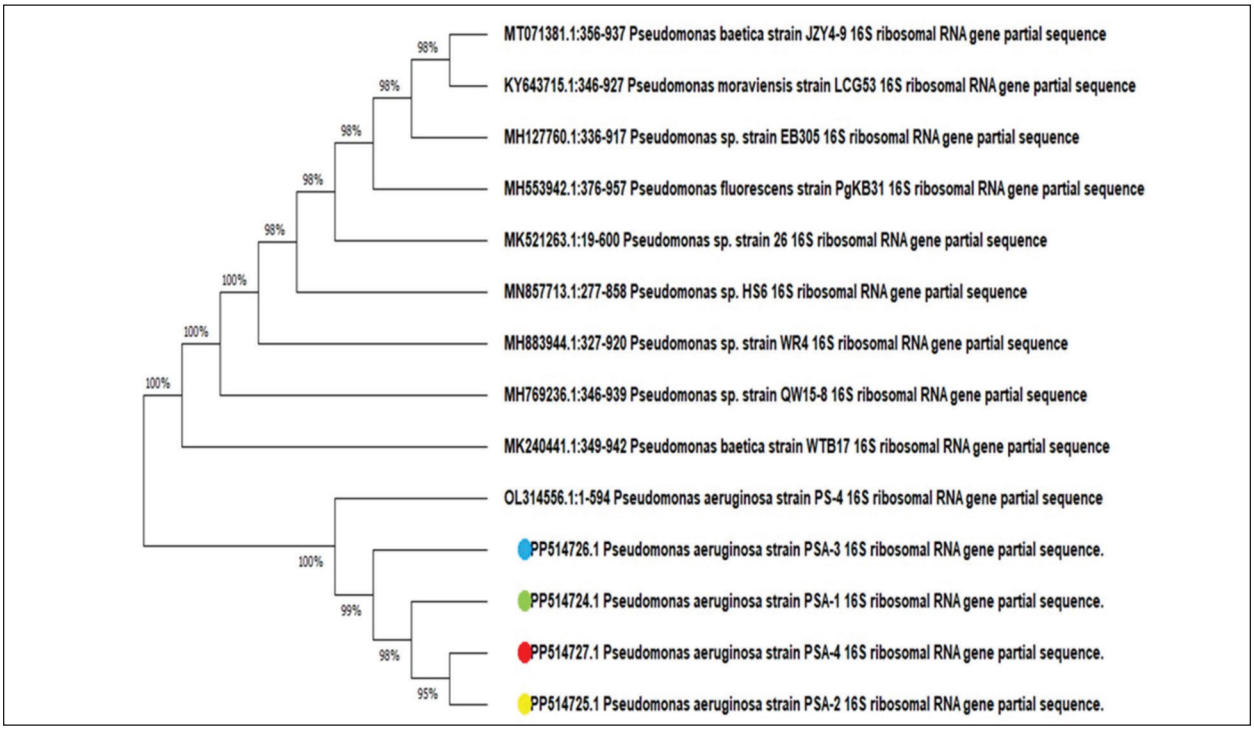

Fig. 6. Phylogenetic tree of *P. aeruginosa* isolated from clinical samples (human, cows, cats, and fish) in comparison with world strains. The tree was constructed using the MEGA X software. The colored dots represent the isolates of the current study.

Acknowledgments

None.

Conflict of interest

All authors of the present study ensure that this investigation has no conflict of interest.

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Self-funded was the only source for this work.

Data availability

All data are provided in the manuscript.

Authors' contributions

All authors participated in all parts of this work.

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