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Research Article

Chemical Synthesis of a Polyvinylpyrrolidone-Capped Silver Nanoparticle and Its Antimicrobial Activity against Two Multidrug-Resistant*Aeromonas* Species

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This study investigated the antimicrobial activity of polyvinylpyrrolidone-capped silver nanoparticles (PVP-AgNPs) *in vitro* against two antibiotic-resistant isolates of *Aeromonas hydrophila* and *A. caviae*. Solid crystal silver nanoparticles were synthesized by the chemical reduction approach employing polyvinylpyrrolidone (PVP) as a neutral stabilizer, glucose as a reducing agent, and sodium hydroxide as a catalyst. The crystallinity and purity of the prepared PVP-AgNPs were investigated using the X-ray diffraction spectrometer (XRD). Transmission electron micrograph analysis demonstrated the formation of irregular spherical shapes PVP-AgNPs with average crystallite size diameters ranging from 5.07 to 9.74 nm. By phenotypic and gene sequence analysis, *A. hydrophila* and *A. caviae* isolates were identified from diseased Nile tilapia. Our findings showed that all *A. hydrophila* and *A. caviae* isolates were multidrug-resistant (MDR) and showed resistance to four antimicrobial classes. The PVP-AgNPs displayed 15 mm and 14 mm clear inhibition zones for *A. hydrophila* and *A. caviae*, respectively. In addition, the minimum inhibitory concentrations for PVP-AgNPs were estimated to be 6.25 μg/ml and 7.5 μg/ml for *A. hydrophila* and *A. caviae*, respectively. The chemically synthesized PVP-AgNPs were found to possess a strong antimicrobial effect against *A. hydrophila* and *A. caviae* with the possibility to be used as a commercialized antimicrobial agent in the aquaculture industry.

1. Introduction

Bacterial pathogens are considered one of the most significant problems hindering the expansion of the global aquaculture industry [1–3]. Motile aeromonads are Gram-negative, motile, facultatively anaerobic bacteria belonging to the family Aeromonadaceae [4–6]. Motile aeromonads are major bacterial pathogens in aquaculture worldwide, which are responsible for mass mortalities among infected farmed fish [7, 8]. These epizootics forced fish farmers to use commercialized, unauthorized, and FDA-unapproved antimicrobial products to control fish mortalities [7]. However, such a massive application of antibiotics could impair human health and deteriorate the aquatic environment [9]. In addition, misuse of antibiotics may enhance the risk of occurrence and transmission of multidrug-resistant bacteria to humans and animals [4]. Therefore, it is crucial to develop innovative methods to control bacterial diseases in aquaculture, such as vaccination, immunostimulants, and nanomaterials [3, 7].

The application of nanoparticles as antibacterial agents in veterinary medicine is a highly dynamic area of research and receives more attention in recent years [10, 11]. Various methods have been used for the synthesis of metallic nanoparticles with unique properties that turn them into useful materials for photocatalysts, adsorbents for heavy metals, biocides, biologically active materials, and sensors [12, 13]. Silver has been considered a safe and nontoxic

inorganic metal. Silver nanoparticles have been widely used in several industrial activities such as textile, medical, cosmetic, and food manufacturing [5, 8, 15]. In addition, silver nanoparticles (AgNPs) showed strong antimicrobial efficiency against many bacterial pathogens infecting farmed animals and allowing alternative controlling of bacterial epidemics. Silver nanoparticles showed strong antimicrobial efficiency agents against several bacterial pathogens including *Vibrio* spp., *Aeromonas salmonicida*, and *Flavobacterium johnsoniae* [15–17]. For the successful synthesis of silver nanoparticles, numerous techniques, including chemical and physical procedures, have been documented [18, 19]. However, most of the compounds employed in these techniques are dangerous substances that are hazardous to both the environment and people. The possibility of using natural substances as reducing agents for the synthesis of nanoparticles is currently being investigated by scientists and researchers. Examples include adding a capping agent (i.e., sodium oleate, polyvinyl alcohol (PVA), polyethylene glycol (PEG), and polyvinylpyrrolidone (PVP) [20], controlling the growth rate, assisting in preventing aggregation, and reducing their size during the preparation of silver nanoparticles [21, 22]. Due to its nontoxicity, biodegradability, biocompatibility, and temperature resistance, PVP is one of the most widely utilized polymeric materials for encasing silver nanoparticles (Figure 1). PVP can act as a carrier for silver nanoparticles in medication delivery systems and silver-containing nanocomposites. Controlling the amount of released silver ions, which varied depending on the materials loaded on AgNPs led to significant antibacterial activity and minimal toxicity [23, 24].

In addition, the capped AgNPs with polyvinylpyrrolidone (PVP-AgNPs) have several unique and attractive features, including reduced interaction with serum protein offering greater antimicrobial activity *in vivo* than other AgNPs. PVP-AgNPs were less toxic to living cells of animal origin or plant [25]. PVP-AgNPs are also found to be well dispersed (stabilized) in water and could be safely applied in the aquaculture industry [26].

In this study, polyvinylpyrrolidone (PVP) was employed as a stabilizer and surface coating (capping) agent during the chemical synthesis of silver nanoparticles. The physical properties of prepared PVP-AgNPs, including shape, crystalline structure, size, and distribution, were investigated. In addition, the antibacterial efficiency of PVP-capped AgNPs against multiantibiotic resistant isolates of *A. hydrophila* and *A. caviae* was estimated using the minimum inhibitory concentration (MIC), and the agar well diffusion method.

2. Materials and Methods

2.1. Fish Sampling. Recurrent mass mortalities were documented among earthen ponds-based Nile tilapia *Oreochromis niloticus* (150–200 g) reared in a private fish farm located in Fayoum governorate. The diseased fish displayed septicemia clinical signs accompanied by anorexia and fin rot. The farm visit revealed the presence of different FDA-unapproved drugs that were used to control fish mortalities in these farms. Sixty fish samples were transported quickly via isothermal boxes with ice to the aquatic laboratory for clinical and bacterial investigations. The collected samples were subjected to an external examination, followed by dissecting to detect any internal lesions.

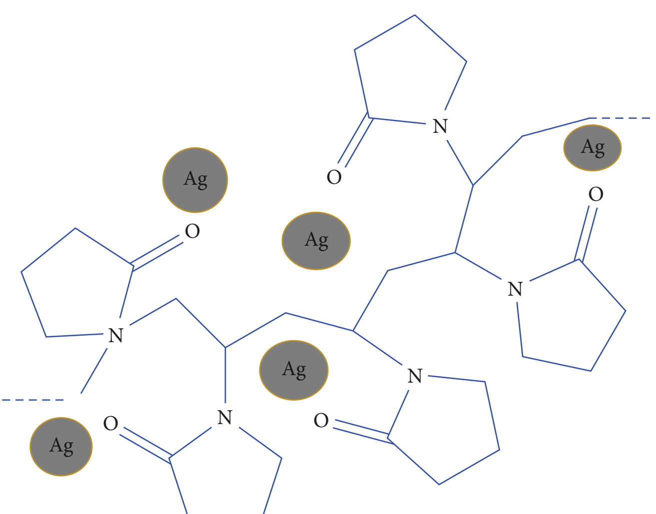

Figure 1: Stabilization of formed AgNPs by PVP.

2.2. Bacteriological Examination and Identification. Suspected bacterial isolates were retrieved from the moribund tilapia's kidneys, spleen, blood, and liver. Loopfuls from these tissues were streaked separately onto tryptic soy agar (Difco) and selective Aeromonas agar base (Biolife) supplemented with ampicillin. The plates were incubated overnight at 30°C for 24 h. The suspected colonies were subcultured onto tryptic soy agar (Difco, Detroit, USA) for purification. The suspected colonies were stained with Gram stain and identified presumptively using the API 20E kit (BioMerieux) following the protocol of the manufacturer. The pure bacterial isolates were stored in sterile aliquots holding tryptic soy broth with 15% (vol/vol) glycerol at −80°C.

2.3. DNA Sequencing. The genomic DNA of the bacterial isolate was extracted using the GeneJET™ Genomic DNA Purification Kit (Thermo Scientific Fermentas, Lithuania, EU), following the manufacturer's protocol for bacterial genomic DNA purification. PCR amplification of the *gyr*B gene was performed using the universal primers, gyrB-3F: 5′-TCCGGC GGTCTGCACGGCGT-3′ and gyrB-14R: 5′-TTGTCCGGG TTGTACTCGTC-3′ [27]. PCR was achieved using the PCR Master Mix of the Maxima Hot Start (Thermo K1051). The process of thermocycling was adjusted at 94°C/5 min (initial denaturation) followed by 30 cycles of 94°C/30 s, 50°C/30 s, and 72°C/1 min and ended at 72°C/10 min. The amplicons were separated by agarose gel electrophoresis with ethidium bromide and visualized using a UV transilluminator. The PCR products were purified using a Gene JET™ PCR Purification Kit (Thermo K0701). Purified PCR products were directly sequenced in both directions by a Bioneer automated sequencer (Bioneer 3730xl, USA), using the same primers of the

PCR process. The raw nucleotide sequences were analyzed, edited, and assembled using the Bio-Edit sequence alignment editor [28]. The assembled sequences were then aligned with nucleotide sequences deposited in the GenBank database. The accession numbers of the current sequences were then obtained. A phylogenetic tree of assembled sequences was constructed using MEGA X [29]. The used parameters were summarized as follows: method: neighbor-joining; model: maximum composite likelihood; bootstrap (1000 replicates); gaps/missing data: pairwise deletion; rates among sites: uniform rates; substitutions included transitions and transversions and pattern among lineages: same (homogeneous).

2.4. Synthesis of PVP-AgNPs. Silver nitrate "$AgNO_3$" and polyvinylpyrrolidone were acquired from Sigma. Glucose and sodium hydroxide were purchased from Acros Organics. Silver nanoparticles were chemically articulated by reducing $AgNO_3$ in a PVP aqueous solution. Glucose was used as a reducer and sodium hydroxide to accelerate the reaction. According to the following method, six grams of PVP and one gram of glucose were added separately to 30 ml of (1M) NaOH solution with vigorous stirring at 60°C. Five ml of silver nitrate solution (1M) was added drop by drop during stirring. After one hour, the colloidal solution was centrifuged and washed several times to collect the prepared AgNPs.

2.5. Characterization of the Prepared PVP-AgNPs. The crystalline structure of the prepared AgNPs was investigated via an X-ray diffraction spectrometer (Panalytical-XPert, Netherlands). The diffraction pattern was detected using generating radiation of 1.5406 Å at a rate of 40 KV. Moreover, the morphology of the prepared silver nanoparticles was determined using a transmission electron microscope (JEM 2100, Japan). Also, Fourier transform infrared FTIR (Perkin Elmer, USA) technique was used to confirm the formation of polyvinylpyrrolidone-capped silver nanoparticles (PVP-AgNPs). KBr disc method in the range of 4000–400 cm^{-1} was applied. In addition, one of the widely used characterization techniques to establish the creation of silver nanoparticles is UV-visible spectroscopy [30]. Concerning Figure 1, the peak present at 406.63 nm (Figure 2) is due to the formation of silver nanoparticles [31].

2.6. Antibiotic Susceptibility. The antibiotic sensitivity of recovered *Aeromonas* species isolates was determined using the disc diffusion method according to CLSI [32]. Briefly, bacterial cultures were inoculated into Mueller–Hinton agar (MHA, Oxoid™), and then the discs of selected antibiotics were distributed on the streaked plates. The measurement of the inhibition zones was determined after 18 h of incubation at 28°C. Ten antibiotics discs were used in this test: oxytetracycline (30 μg), sulfamethoxazole-trimethoprim (23.75 μg/1.25 μg), cephalexin (30 μg), neomycin (10 μg), imipenem (10 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), kanamycin (30 μg), florfenicol (30 μg), and gentamicin (10 μg). The investigated isolates of *Aeromonas* species were recognized as multidrug-resistant (MDR) when these isolates were resistant to three or more antimicrobial categories [33].

2.7. In Vitro Antimicrobial Activity of PVP-AgNPs

2.7.1. Disc Diffusion Method. Fresh *A. hydrophila* and *A. caviae* overnight cultures were streaked onto Muller–Hinton base agar. 50 μl of PVP-AgNPs were applied separately to sterile filter paper discs (6 mm). The impregnated discs were left to dry for a day before each experiment. The discs were tested for their antibacterial sensitivity by placing them on Muller–Hinton agar plates and incubated at 28°C for 24 h. The inhibition zone that appeared around the disc was measured and recorded [34].

2.7.2. Minimum Inhibitory Concentration (MIC). The tube double dilution method was performed. Fresh *A. hydrophila* and *A. caviae* cultures were incubated in nutrient agar broth overnight at 28°C. The cell density was adjusted to 10^6 CFU/ml. Double-fold serial dilution of prepared PVP-AgNPs was performed starting from the concentration of 100 μg/ml. A series of concentrations of the prepared PVP-AgNPs with equal volumes were added to the bacterial solution. The tubes were incubated for 24 h at 28°C, after that 100 μl from each dilution tube was plated in MHA (Muller–Hinton agar plates) and incubated overnight at 28°C. Bacterial growth was determined by visual inspection of the bacterial culture. The lowest concentration of the antibacterial agent at which no bacterial growth was observed in the culture suspensions was defined as the MIC [35].

3. Results and Discussion

3.1. Clinical Examination. The recurrent mass mortalities of cultured Nile tilapia are common in this farm and adjacent farms. Moribund tilapia exhibited the typical signs of septicaemia, including surface haemorrhagic patches, darkening, scales detachment, and ascites. Internally, the necropsied fish displayed hemorrahges and congestion of the spleen, liver, kidney, gonads, and gut. In addition, a distended gall bladder was also noticed among infected fish, denoting that septicemic bacterial pathogens could be incriminated in such epizootics. These results agree with those of Abu-Elala et al. [4] and Sherif et al. [8]. Bacterial diseases are responsible for around 50% of farmed fish mortalities caused by infectious diseases [36].

3.2. Bacterial Identification. The bacteriological investigation revealed the presence of green colonies with dark centers. All bacterial strains were Gram-negative, motile, rod shape, and positive for both the oxidase and catalase tests. The bacterial strains were recognized as *A. hydrophila* and *A. caviae* based on the data of API20 E and the sequencing of the B-subunit of DNA gyrase, a type-II DNA topoisomerase (*gyr*B) gene. The sequencing of *gyr*B is regarded as an important phylogenetic marker for the identification of

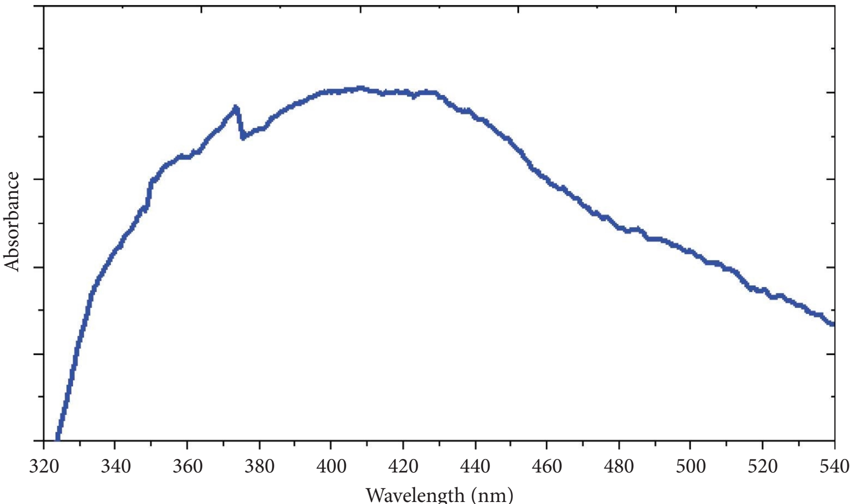

Figure 2: UV-vis spectra of the prepared PVP-AgNPs.

Aeromonas spp. [4] and provided strong discriminatory power among *Aeromonas* spp. [37].

The BLAST analysis of the *gyr*B gene confirmed that the six strains are deeply embedded in the Aeromonadaceae family, and their identity was confirmed as *A. hydrophila* (three isolates), and *A. caviae* (three isolates). The alignment of the accession numbers of the current *A. hydrophila* (OP485626- OP485627- OP485628) showed a high similarity (99.72–97.94%) among the following sequences of typing isolates of *A. hydrophila* (AB436660^T, CP000462^T, JN711797^T, LC644209, JQ234895, and OM965641). The BLAST analysis of the accession numbers of the current *A. caviae* (OP822676, OP822677, and OP822678) showed a high similarity (99.90–98.49%) among the following sequences of *A. caviae* (AJ868400^T, MK572675, LC644319, MT371973, and MT371974). The phylogenetic analysis exhibited that the current *A. hydrophila* and *A. caviae* isolates, which incriminate in recurrent mortalities in farmed tilapia, are separated into two different branches. The first one included *A. caviae* of this study grouped with other accession numbers of typing and nontyping strains of *A. caviae* with a high bootstrap value of 100%. The second branch included *A. hydrophila* of this study grouped with other accession numbers of typing and nontyping strains of *A. hydrophila* with a high bootstrap value of 99%. In addition, the sequences of *A. hydrophila* and *A. caviae* are quite distinct from other members of the family Aeromonadaceae (Figure 3).

3.3. Multidrug Resistance of A. hydrophila. In the current study, *A. hydrophila* and *A. caviae* isolates revealed higher resistance levels to the ten antibiotics examined. All tested *A. hydrophila* isolates were multidrug-resistant (MDR) and showed resistance to at least four antimicrobial categories, including oxytetracycline, sulfamethoxazole-trimethoprim, gentamicin, cephalexin, and neomycin. While all *A. caviae* were also MDR and showed similar results of resistance except two isolates showed susceptibility to gentamicin, *A. hydrophila* and *A. caviae* isolates were sensitive to imipenem, florfenicol, kanamycin, and ciprofloxacin. Two *A. hydrophila* isolates demonstrated intermediate susceptibility to nalidixic acid. While two isolates of *A. caviae* showed resistance to nalidixic acid. These results agreed with the resistance profiles of *A. hydrophila* and *A. caviae* documented in several studies in Egypt [4, 38]. The wide expansion of multidrug resistance bacteria in fish farms, veterinary, and human medicine is a serious growing problem and provokes health hazard issues. Antibiotics are widely applied in veterinary practices for the control of significant bacterial pathogens after performing the antibiotics susceptibility test. However, the application of several unapproved antimicrobial agents in aquaculture has been responsible for the existence and transmission of multidrug-resistant bacteria to humans and animals [4]. In addition, the extensive use of antibiotics for long-periods might diminish the efficiency of these antibiotics on fish farms [5]. Consequently, the emergence of fish diseases caused by antibiotic resistance bacteria enforced investigators to synthesize several nanomaterials with antibacterial activity [12, 13].

3.4. Synthesis and Characterization of PVP-AgNPs. The crystallinity and average particle size of the prepared AgNPs were determined using X-ray diffraction (XRD) measurements. Figure 4 shows different peaks at 38.45°, 46.35°, 64.75°, and 78.05° assigned to (111), (200), (220), and (311), respectively. The previous XRD pattern confirmed the face-centered structure of the prepared silver nanoparticles that matched the reference data of AgNPs (No. 19-0629). The mean diameter of the prepared AgNPs (D) is inversely proportional to the full width at the half maximum value (β) and can be calculated [39, 40] from the Debye–Scherrer equation:

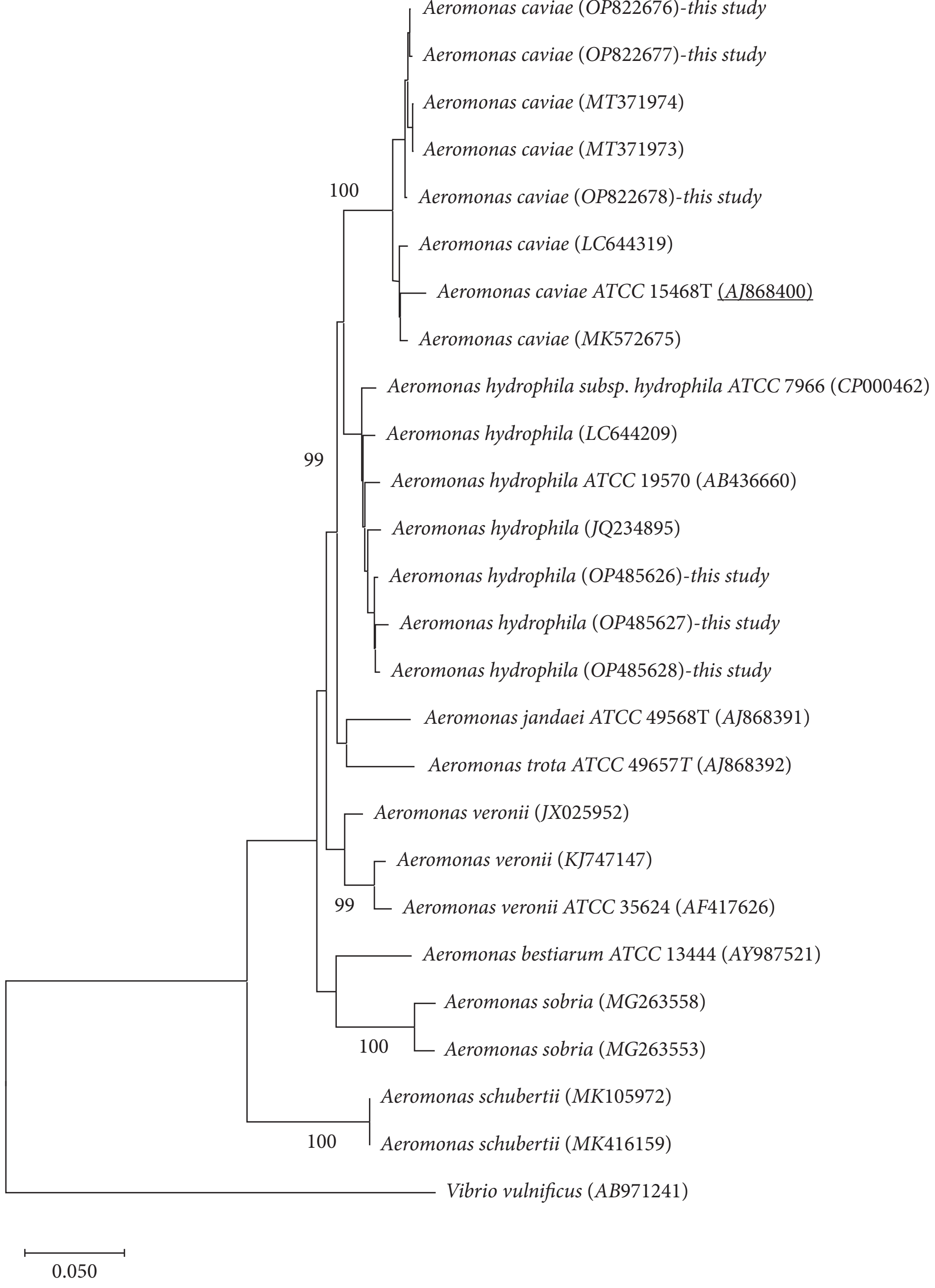

Figure 3: Phylogenetic tree revealed the comparative alignment analysis of the *gyr*B gene sequenced from *A. hydrophila* and *A. caviae* isolated from moribund tilapia.

$$D = \frac{k\lambda}{\beta \cos\theta}, \quad (1)$$

where λ is the wavelength of the radiation source, k is the Scherrer constant (0.9, −1), and θ is the half diffraction of the Bragg angle. The most intense peak (111) in the XRD pattern of AgNPs (Figure 4) is used to calculate the mean diameters of the prepared nanoparticles. The average particle size was found to be matched with the average particle size obtained from TEM measurements.

The morphology and particle size of the AgNPs were determined by the TEM technique. Figure 5 shows an irregular spherical shape with an average diameter ranging from 5.07 to 9.74 nm.

Figure 6 shows the FTIR spectra of pure PVP and PVP-AgNPs samples. Concerning PVP spectra, the characteristic peak at 35236 cm^{-1} was attributed to the stretching vibration of the OH group. The symmetric stretching vibration of CH_2 and C-H was detected at a wavenumber of 2920 and 2890 cm^{-1}, respectively. Furthermore, peaks of the asymmetric stretching vibration of CH_2 in the pyrrole ring were located at 2955.90 cm^{-1}. The peak at 1653.52 cm^{-1} was assigned to the C=O starching vibration. Also, peaks characteristic of the C-N bending mode

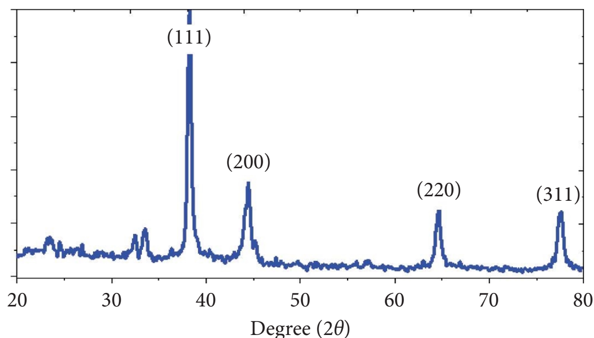

Figure 4: XRD spectra of the prepared PVP-AgNPs.

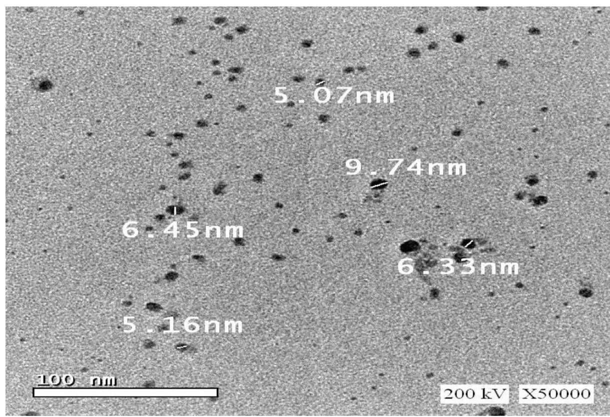

Figure 5: TEM image of PVP-AgNPs.

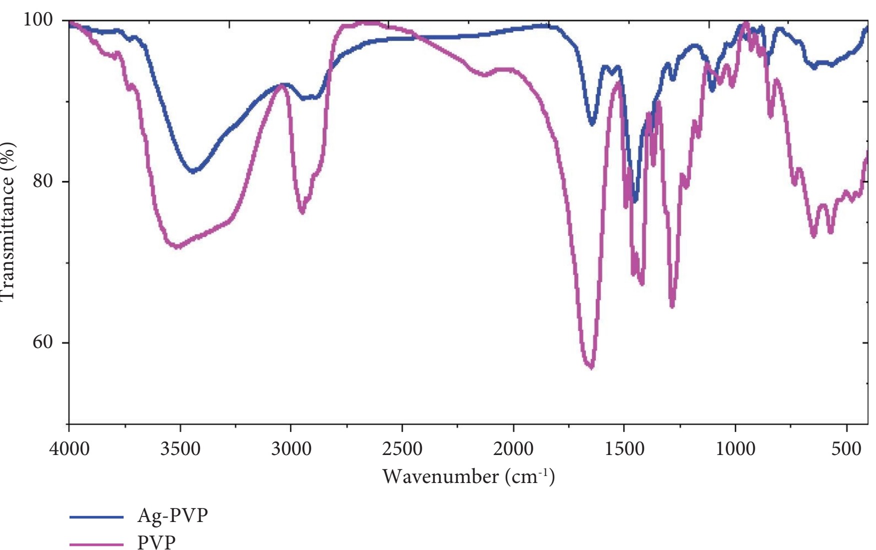

Figure 6: FTIR spectra of PVP and PVP-AgNPs.

were detected at 1019 and 1073 cm^{-1}. Additionally, peaks at 1289 and 574 cm^{-1} were due to N-OH and N-C=O bonds, respectively [41]. A slight shift in peak position and intensity was observed in the spectra of PVP-AgNPs (see Figure 3), with the peaks related to the C=O and O-H stretching being moved to 1649 and 3448 cm^{-1}, respectively. The peak strength of the N-OH and N-C=O bands was also reduced, indicating that the PVP joined to AgNPs via nitrogen atoms due to the easy donation of nitrogen lone pair electrons.

The chemical reduction method is the most common way to synthesize silver nanoparticles due to its simplicity, lower reaction temperature, and solubility of prepared AgNPs in water [18, 42]. Silver nanoparticles could be synthesized by the chemical reduction method using polyvinylpyrrolidone (PVP) as a neutral stabilizer, glucose as a reducing agent, and sodium hydroxide as a catalyst [23, 24]. Polyvinylpyrrolidone is commonly used as a great stabilizing agent, surfactant agent, reducing agent, nanoparticle dispersant, and shape-directing agent in the chemical synthesis of silver nanoparticles [22]. Recent studies proved that PVP-AgNPs had long stability features than other AgNPs and might stay stable for almost one month [26]. PVP directly could form a protective layer on the surface of silver nanoparticles to avoid the aggregation of nanoparticles. Therefore, PVP-AgNPs could be used for long periods after synthesis [26]. Interestingly, PVP-capped AgNPs were found to be less susceptible to the changing surface charge by pH change, hardness, the concentration of dissolved organic matter, and ionic strength [43, 44]. In addition, the capped AgNPs with polyvinylpyrrolidone minimized the interaction with a biological liquid such as serum protein providing higher antimicrobial activity *in vivo* than other uncapped or capped AgNPs. Moreover, PVP-AgNPs were found to be less toxic to plant and mammalian cells [25]. Finally, PVP-AgNPs are found to be well dispersed in water with several potential applications in the aquaculture industry [26].

3.5. In Vitro Antimicrobial Activity of PVP-AgNPs. The results of the MIC assay indicated that the lowest concentrations of PVP-AgNPs that visually inhibited the growth of A. hydrophila and A. caviae were 6.25 μg/ml and 7.5 μg/ml, respectively. The results were confirmed by disc diffusion experiments on Müller–Hinton agar plates. Silver nanoparticles demonstrated strong antibacterial activities against tested *A. hydrophila* followed by *A. caviae* with inhibition zone of 15 mm and 14 mm, respectively (Figure 7). To estimate whether the chemically synthesized PVP-AgNPs would be a promising antibacterial agent, we analyzed the antibacterial activities of the prepared PVP-AgNPs from two aspects as follows: MIC and the inhibition zone test. The important aquatic pathogens, *A. hydrophila,* and *A. caviae* were tested. As shown in Figure 3 the chemically synthesized PVP-AgNPs exhibited excellent activity against tested pathogenic bacteria. PVP-AgNPs express their antimicrobial activity in many ways. They disrupt the bacterial cell membrane, generate reactive oxygen species (ROS), bind with microbial DNA, and interfere with protein synthesis. The AgNPs are effective against multidrug resistant bacteria (MDR). The release of silver particles is one mechanism, silver may bind to bacterial cell membrane proteins disrupting the membrane and leading to the death of the bacterial cells. Intracellularly, it may bind to cytochrome and nucleic acids, damaging them and inhibiting cell division.

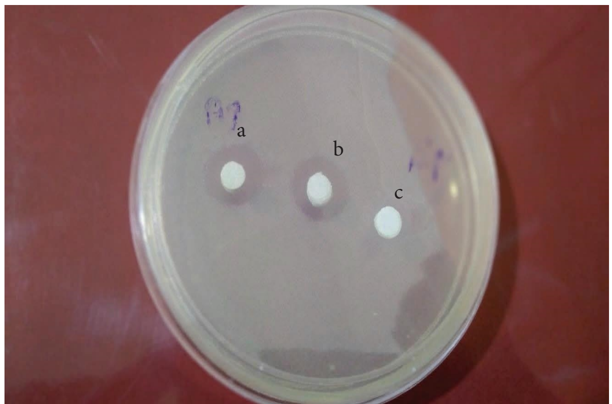

Figure 7: The disk diffusion method showed the antimicrobial activity of PVP-AgNPs against *A. hydrophila* (a), and *A. caviae* (b), and the blank disc (c) is used for the control negative.

4. Conclusion

Aeromonas hydrophila and *A. caviae* are important bacterial pathogens that are responsible for serious high mortality among cultured Nile tilapia. The massive use of antimicrobial drugs to control bacterial diseases facilitates the transmission of resistant bacterial isolates in the aquatic environment. PVP-AgNPs demonstrated potent antimicrobial activity in vitro against *A. hydrophila* and *A. caviae* isolates. The physical properties of prepared PVP-AgNPs revealed that size distribution and irregular spherical shapes might promote the strong stability of PVP-AgNPs. These results proved that PVP-AgNPs have several advantages that encourage their applications as an antibacterial agent in aquaculture.

Data Availability

All required data are included in this article.

Ethical Approval

The study followed the guidelines of the Institutional Animal Care and Use Committee, National Research Centre, Egypt.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

This study was conducted with the cooperation of all authors.

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