

# Molecular detection of multidrug-resistant *Pseudomonas aeruginosa* of different avian sources with pathogenicity testing and in vitro evaluation of antibacterial efficacy of silver nanoparticles against multidrug-resistant *P. aeruginosa*

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**ABSTRACT** *Pseudomonas aeruginosa* (*P. aeruginosa*) is a serious zoonotic pathogen threaten the poultry industry causing severe economic losses therefor, this study aimed to isolation, phenotypic, molecular identification of *P. aeruginosa* from different avian sources (chickens, turkey, pigeons, table eggs, and dead in shell chicken embryos), from different Egyptian governorates (Giza, Qalubia, Beheira, El-Minya, and Al-Sharqia) with applying of antibiotic sensitivity test on all *P. aeruginosa* isolates. Highly resistant isolates ( $n = 49$ ) were subjected to molecular identification of *P. aeruginosa* with detection of resistant genes including carbapenemase-encoding genes blaKPC, blaOXA-48, and blaNDM. On the base of molecular results, a highly resistant *P. aeruginosa* strain was tested for its pathogenicity on day old specific pathogen free (SPF) chicks. Also, in vitro experiment was adopted to evaluate the efficacy of silver nanoparticles (Ag-NPs) against highly antibiotic-resistant *P. aeruginosa* strains. The overall isolation percentage was from all examined samples

were 36.2% (571/1,576) representing 45.2% (532/1,176) from different birds' tissues and 39/400 (9.7%) from total egg samples. Some of isolated strains showed multi-drug resistance (MDR) against kanamycin, amoxicillin, amoxicillin-clavulanic acid, neomycin, chloramphenicol, vancomycin, cefotaxime clavulanic acid, lincomycin-spectinomycin, co-trimoxazole, cefoxitin, gentamycin, and doxycycline. These MDR strains were also molecularly positive for ESBL and carbapenemase-encoding genes. MDR strain showed high pathogenicity with histopathological alterations in different organs in challenged birds. Main histopathological lesions were necrosis of hepatocytes, renal tubular epithelium, and heart muscle bundles. The MDR strain showed in vitro sensitivity to Ag-NPs. In conclusion, MDR *P. aeruginosa* is a serious pathogen causing high morbidity, mortality, and pathological tissue alterations. Ag NPs revealed a promising in vitro antimicrobial sensitivity against MDR *P. aeruginosa* and further in vivo studies were recommended.

**Key words:** antibiotic resistance, histopathology, pathogenicity testing, resistant gene, silver nanoparticle

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## INTRODUCTION

*Pseudomonas aeruginosa* is a serious pathogen affecting most avian species at any age causing severe economic losses in the poultry sector, it is considered as a bacterial hazard that contaminated hatcheries and induces severe respiratory signs, enteritis, septicemia, keratitis, sinusitis, omphalitis, rapid morbidity, embryonic death, as well as mortalities in all ages (Walker et al.,

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2002; Shukla and Mishra, 2015; Algammal et al., 2023). In case of experimental infection, *P. aeruginosa* induced mortalities about 70% when inoculated via subcutaneous route in 3-days-old chicks. Mortalities of 90 and 100% were recorded after dipping or inoculation in yolk sac of 17-days-old embryonated chicken eggs, respectively (Bakheet and Torra, 2020). The haphazard treatments resulting in multiple drug resistance of most *P. aeruginosa* strains as they are highly susceptible to genetic modifications (Odoi et al., 2021). Besides, they can produce biofilm (Channa et al., 2019; Shahat et al., 2019). *P. aeruginosa* is a gram-negative, motile, non-spore-forming rods, produce watery soluble green pigment with a specific fruity odor (Dinev et al., 2013; Elsayed et al., 2016; Eman et al., 2017). *P. aeruginosa* has attracted attention as a dangerous pathogen for public health, causing a variety of infections in humans and food animals, and carrying multidrug resistance characteristics that can be passed on to other human and animal pathogens (Shahat et al., 2019). Antimicrobial resistance is currently one of the most prominent public health issues and it is on the rise in developing countries (Le Guern et al., 2023). Indeed, there is mounting evidence that animals serve as a reservoir for antimicrobial resistance (Abd El-Hack et al., 2022). In gram-negative bacteria, synthesis of ampC-lactamases, carbapenemases, and extended spectrum lactamases (ESBL) is the main mechanisms via which resistance is mediated (Schill et al., 2017). These enzymes' genes are frequently found on plasmids that also contain resistance genes to other commonly used antibiotics in clinical settings (Egorov et al., 2018). Infections with these multidrug-resistant organisms (MDROs) will thus pose therapeutic challenges, the antibiotic pipeline is drying up, and no new antimicrobial agents to treat infections caused by MDROs are expected in the near future (Bettiol and Harbarth, 2015; Dougnon et al., 2023). Antibiotics are commonly used in veterinary medicine for both therapeutic and prophylactic purposes and this is prohibited by the European Union (Jawher and Hasan, 2023). However, some countries especially in North America still use it as growth promoters (Economou and Gousia, 2015). In recent years, the prevalence of gram-negative bacteria producing ESBLs and carbapenemase has been widely reported in food-producing animals (Haenni et al., 2016). Therefore, it is critical to identify *P. aeruginosa* susceptibility pattern and detect it precisely and easily avoid antibiotics that are ineffective (Haleem et al., 2011; Hamisi et al., 2012). Nanotechnology applications in diagnosis and treatment of

poultry diseases have been studied in the literature (Abd El-Ghany et al., 2021). The nanoparticles, which are small, engineered particles in the size of 1 to 100 nm, have emerged as a potential alternative to antibiotics especially in the cases of multidrug-resistant bacteria (Shaalan et al., 2016; Lang et al., 2021). With special regard to silver nanoparticles, potent antibacterial activity against gram-negative bacteria was recorded (Farouk et al., 2020; Elgendy et al., 2022). In this study, a trial to isolate *P. aeruginosa* from birds and eggs, performed the bacterial identification, antibacterial sensitivity test and molecular characterization of the bacteria with detection of resistant genes. Pathogenicity study will be conducted with monitoring of morbidity and mortality rates and histopathological lesions besides, a trail to synthesis and characterize silver nanoparticles and test them in vitro for antibacterial activity against the isolated bacteria.

## MATERIALS AND METHODS

This work was conducted according to ARRIVE 2.0 guidelines and ethically approved by The Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine Cairo University with code VETCU23052022444.

### Isolation and Identification of *Pseudomonas Aeruginosa*

**Sample's Collection** Samples (3 freshly dead birds/farm) were collected from broiler chicken, turkey, and pigeons' farms suffered from high mortalities at first 3 wk of rearing as well as, 20 commercial table eggs were collected from each governorate (5 eggs/market), and 20 dead in shell chicken embryos (5 eggs/hatchery) were collected from each of the investigated Egyptian governorates as summarized in Table 1. Samples were collected, labeled, and transported on ice box for further investigations in the laboratory of Microbiology Department, Faculty of Veterinary Medicine, Cairo University.

**Tissue Samples** Under complete hygienic conditions, heart, liver, kidney, air sac, unabsorbed yolk sac (if found), and subcutaneous swabs (in case of facial edema or cellulitis) were collected separately from each examined bird as well as, swabs were collected from external and internal content each egg separately as summarized in Table 2. Samples  $n = 1,576$  representing (1,176 bird tissue sample and 400 egg samples) were inoculated in nutrient broth and incubated aerobically at 37°C for

**Table 1.** Number of examined poultry samples in different Egyptian governorates.

Governorate	Chicken farms (1–21-days old)	Turkey farms	Pigeon farms	Commercial table eggs	Dead in shell chicken embryos
Giza	35	1	2	20	20
Qalubia	18	5	2	20	20
Beheira	8	1	0	20	20
El- Minya	6	3	0	20	20
Al-Sharqia	5	2	1	20	20
Total	72	12	5	100	100

**Table 2.** Number of examined tissue samples.

Tissue source	Heart	Liver	Kidney	Air sac	Yolk sac	Subcutaneous tissue swabs	External shell swabs	Internal egg content
Chicken (72 × 3) = 216	216	216	216	216	80	20	0	0
Turkey (12 × 3) = 36	36	36	36	36	0	8	0	0
Pigeon (5 × 3) = 15	15	15	15	15	0	0	0	0
Table eggs (100)	0	0	0	0	0	0	100	100
Dead in shell embryos (100)	0	0	0	0	0	0	100	100
Total	267	267	267	267	80	28	200	200

24 h. A loopful from growth was streaked on Cetrinide agar, *Pseudomonas* agar and MacConkey agar then incubated under aerobic condition at 37°C for 24 h.

**Gram Staining** The isolated bacteria ( $n = 571$ ) were characterized morphologically using Gram's staining method as described by (Merchant and Packer, 1967).

**Biochemical Identification** The bacteria that showing characteristics colonial morphology of *P. aeruginosa* ( $n = 571$ ) were subjected to biochemical test such as sugar fermentation (glucose and mannitol), oxidase, indole, catalase, MR, VP, motility test, citrate, urease, nitrate reduction, and gelatin hydrolysis according to Barrow and Feltham (2003), also all isolates were examined for growth pattern on MacConkey agar at 4°C and 42°C under aerobic condition.

**Antibiotic Sensitivity Test** All isolated bacteria ( $n = 571$ ) were tested for antibiotic susceptibility using Kirby Bauer disk diffusion method (Bauer et al., 1966), Muller Hinton broth, Muller Hinton agar, and antibiotic disks are used (kanamycin K<sub>30</sub> µg, amoxicillin Aml<sub>10</sub> µg, amoxicillin-clavulanic acid AX<sub>25</sub> µg, neomycin N<sub>10</sub> µg, chloramphenicol C<sub>30</sub> µg, vancomycin VA<sub>30</sub> µg, cefotaxime clavulanic acid CTC<sub>40</sub> µg, lincomycin-spectinomycin LS<sub>109</sub> µg, co-trimoxazole COT<sub>25</sub> µg, cefoxitin CX<sub>30</sub> µg, gentamycin GEN<sub>10</sub> µg, and Doxycycline DO<sub>5</sub> µg). The results were interpreted according to NCCLS (2002).

**Molecular Characterization of *P. Aeruginosa*** Genomic DNA extraction was performed using a boiling method for 49 highly resistant *P. aeruginosa* isolates. Specific primers targeting the genes oprL (F: ATg-gAAATgCTgAAATTCggC and R: CTTCTTCAgCTC-gACgCgACg) were used to amplify 504 bp products (Chand et al., 2021). Following optimization, the following reaction mixtures (25 µL) were set up: 5 µL of DNA template, 0.5 µL of each set of primers, 12.5 µL of 2× PCR Master Mix, and 6.5 µL DNase/RNase-free water. The reaction mixtures were treated to the following thermal cycling conditions: 94°C for 5 min, then 30 cycles lasting 1 min each at 94°C, 55°C, and 72°C, followed by a final extension lasting 10 min at 72°C. Using GeneRuler 100 bp DNA Ladder and a 1.5% agarose gel, electrophoresis was carried out.

**Molecular Detection of Resistant Genes** A total of 49 highly resistant *P. aeruginosa* isolates on the base of antibiotic sensitivity test were chosen, and the carbapenemase-encoding genes blaKPC, blaOXA-48, and blaNDM were subsequently examined. The gene-specific PCR primers provided in Table 3 were used to amplify these gene fragments. One microliter of genomic DNA

template, 12.5 µL of DNA polymerase master mix (Takara Bio Inc., Shiga, Japan), and 0.4 µM of each primer made up the PCR mixes, which had a total reaction volume of 25 µL. The following cycling parameters were used for the blaKPC, blaOXA-48, and blaNDM PCR amplifications: 30 cycles with a 1-min denaturation step at 94°C, a 1-min annealing step at 55°C, a 2-min extension step at 72°C, and a final single 10-min extension step at 72°C.

The genes for ESBLs were examined in all positive *P. aeruginosa* isolates. For the purpose of identifying the resistance determinant genes SHV, TEM, and CTX-M, multiplex polymerase chain reaction (PCR) was carried out utilizing particular oligonucleotide primers (Table 4). PCR mixtures of 25 µL total volume were prepared from 12.5 µL Emerald Amp GT PCR master mix (Takara Bio Inc., Shiga, Japan), 0.5 µL from each primer with concentration of 10 pmol, 3 µL template DNA from each isolate, and 5.5 µL water. The following conditions were used to amplify all reactions: initial denaturation at 95°C for 15 min; 30 cycles of 94°C for 30 s, 62°C for 90 s, and 72°C for 60 s; and finally, a final extension at 72°C for 10 min. The PCR products were electrophoresed on 1.5% agarose gel, a 100-bp DNA ladder.

**Table 3.** Primer sequences for carbapenemase-encoding genes (Elshafie et al., 2019).

Gene	Sequence	Size (bp)
<i>blaKPC</i>	F:ATGTCACGTATCGCCGTCT R:TTTTGAGAGCCTTACTGCC	882
<i>blaOXA-48</i>	F:TTGGTGGCATCGATTATCGG R:GAGCACTTCTTTTGTGATGGC	743
<i>blaNDM</i>	F:GGTTTGGCGATCTGGTTTTC R:CGGAATGGCTCATCACGATC	621

**Table 4.** Primer sequences for ESBL-encoding genes (Monstein et al., 2007).

Gene	Sequence	Size (bp)
<i>blaTEM</i>	F: CGC CGC ATA CAC TAT TCT CAG AAT GA R: ACG CTC ACC GGC TCC AGA TTT AT	445
<i>blaSHV</i>	F: CTT TAT CGG CCC TCA CTCAA R: AGG TGC TCA TCA TGG GAA AG	237
<i>blaCTXM</i>	F: ATG TGC AGY ACC AGTAAR GTK ATG GC R: TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593

**Pathogenicity Testing** On the base of antibiotic sensitivity test against the selected antibiotics, the highly resistant strains were selected and then subjected to molecular detection of the resistant genes and the strain that showed highly genetic resistant against all the tested resistant genes was selected to pathogenicity testing.

Thirty, 1-day-old SPF chicks from the SPF Farm, Koum Oshein, El-Fayoum, Egypt were divided into 3 groups 10 chicks in each as follow; Group 1: each chick was injected S/C with 0.25 mL fresh nutrient broth containing  $1.5 \times 10^8$  CFU/mL (0.5 McFarland tube which is confirmed by plate count) of molecularly identified MDR *P. aeruginosa* strain, Group 2: each chick was inoculated orally with 0.5 mL nutrient broth containing  $1.5 \times 10^8$  CFU/mL (0.5 McFarland tube) of MDR *P. aeruginosa* strain by crop gavages for 2 successive days, and Group 3: kept as control negative noninfected group. Birds were kept in the laboratory units of Microbiology Department, Faculty of Veterinary Medicine, Cairo University, and birds were reared on 3 separate pens under controlled environmental conditions and birds supplied with sterile water and autoclaved starter commercial balanced ration ad libitum.

**Morbidity and Mortality Rates** Daily observation of clinical signs and mortalities were adopted all over the experimental period (2 wk).

**Sampling** Tissue samples (liver and heart) were collected from all freshly dead birds for reisolation. Also, at 5-days postinfection (7-days old), 3 chicks per group were ethically slaughtered to collect tissue samples form (liver and heart) for reisolation as well as from (kidneys, liver, and heart) for further histopathological examination.

**Histopathological Examination** Samples from kidneys, liver, and heart were fixed in neutral buffered formalin (NBF) 10% for 24 h, then trimmed, processed, embedded, and sectioned by automated microtome at a thickness of 5  $\mu$ m. Tissue sections were mounted and stained with H&E stain (Bancroft and Gamble, 2008). Images were taken by a digital camera attached to a light microscope.

## In Vitro Assessment of Silver Nanoparticles Efficacy Against Resistant Strains of *P. Aeruginosa*

**Inoculum Preparation** A molecularly identified *P. aeruginosa* highly antibiotic-resistant strain was selected. The selected strain was inoculated into nutrient broth and incubated under aerobic conditions at 37°C for 18 h and the resulted growth was adjusted at a concentration of  $10^6$  CFU/mL.

**Silver Nanoparticles Preparation and Characterization** Silver nanoparticles were produced via chemical reduction of silver nitrate. The method utilized both sodium citrate and sodium borohydride as reducing agents with coating of the produced particles with polyvinylpyrrolidone as described by Shaalan et al. (2020). The produced nanoparticles were characterized by transmission electron microscopy imaging to define shape and size.

**Minimal Inhibitory Concentration Assay** The synthesized silver nanoparticles were evaluated for the minimal inhibitory concentration (MIC) value. The assay was performed in triplicates according to Shaalan et al. (2017). Freshly prepared bacterial culture of *P. aeruginosa* ( $10^6$  CFU/mL) was incubated with different silver nanoparticles concentrations (25, 12.5, 6.25, and 3.13  $\mu$ g/mL). After 24 h incubation, 100  $\mu$ L of each concentration was streaked on Müller Hinton agar plate and incubated overnight. MIC value was determined based on the concentration at which no bacterial growth was detected on the agar plate.

## RESULTS

### Isolation and Identification of *P. Aeruginosa*

As presented in Tables 5 and 6, the positive colonies appeared as green fluorescence and a few colonies appeared as brownish colonies on the surface of Cetrimide agar. The total isolation percentages from all

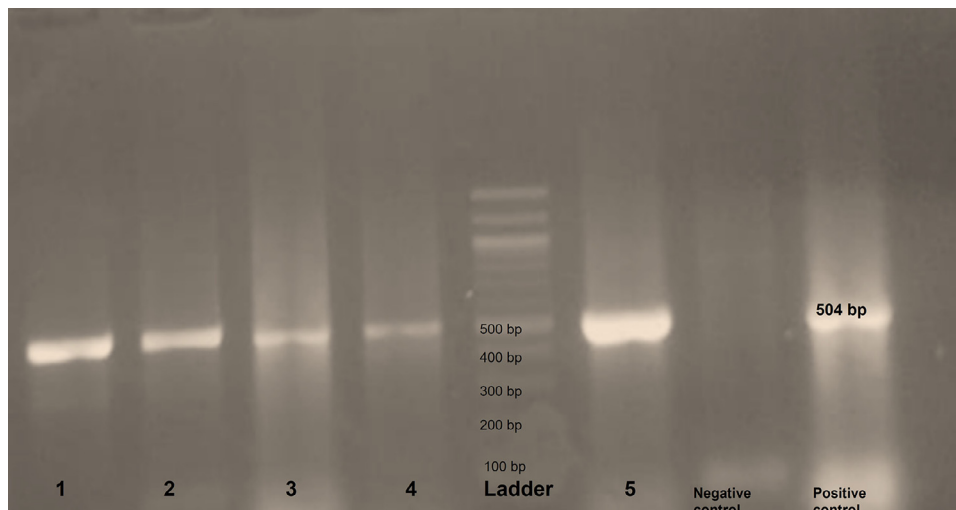
**Table 5.** Isolation percentage from different examined tissue samples.

No. of examined birds	Heart	Liver	Kidney	Air sac	Yolk sac	S/C swabs	Total positive
Isolation results							
Chicken ( $n = 216$ )	112/216 (51.8%)	109/216 (50.4%)	43/216 (20%)	110/216 (51%)	76/80 (95%)	14/20 (70%)	464/964 (48.13 %)
Turkey ( $n = 36$ )	21/36 (58.3%)	16/36 (44.4%)	6/36 (16.6%)	2/36 (5.5%)	-	6/8 (75%)	51/ 152 (45%)
Pigeon ( $n = 15$ )	7/15 (46.6%)	6/15 (40%)	4/15 (26.75)	-	-	-	17/ 45 (37.7%)
Total bird samples ( $n = 267$ )	140/267 (52.4%)	131/267 (49%)	53/267 (19.8%)	112/252 (44.4%)	76/80 (95%)	20/28 (71%)	532 / 1176 (45.2%)

**Table 6.** Isolation percentage eggs.

Samples	Isolation rate		Total isolation rate from eggs
	External eggshell swabs	Internal egg contents	
Table eggs (100)	-	10/100 (10%)	39/400 (9.7%)
Dead in shell embryos (100)	4/100 (4%)	25/100 (25%)	
Total egg samples	4/200 (2%)	35/200 (17.5%)	





**Figure 1.** Photo of the agarose gel (1%) electrophoresis of PCR assay for the oprL gene, samples in lanes from 1 to 5 are samples showing positive bands at 504 bp.

examined samples were 36.2% (571/1,576). In chickens, 464 tissue samples out of 964 were positive for *P. aeruginosa* with isolation percentage (48.1%), followed by turkey 51 tissue samples were positive out of 152 (45%) and in pigeons, 17 samples were positive out of 45 (37.7%) tissue samples.

The total isolation rate of *P. aeruginosa* from eggs was 9.7% (39/400). In table eggs *P. aeruginosa* isolation percentage was 10% from internal egg contents while, in dead in shell embryos isolation percentage was 4% (4/100) from the external eggshell swabs and 25% (25/100) from internal egg contents.

The primary bacteriological isolation percentage from different tissues of total birds 267 (216 chicken, 36 turkey and 15 pigeons) was 52.4, 49, 19.8, 44.4, 95, and 71.4% from heart, liver, kidney, air sac, yolk sac, and S/C swabs, respectively.

Gram staining of the suspected colonies showed the presence of gram-negative rods. The suspected greenish colonies were further subjected to biochemical identification and positive isolates glucose fermentation (negative), mannitol fermentation (positive), oxidase (positive), indole (negative), catalase (positive), MR (negative), VP (negative), motility test (motile), citrate (positive), urease (negative), nitrate reduction (positive), gelatin hydrolysis (positive), and Cetrimide test (positive).

All positive isolates were further subjected to antibiotic sensitivity testing and the results of the antibiotic sensitivity of *P. aeruginosa* was tested by using disk diffusion method against different antimicrobials, showed that the high resistance (100%) was noticed against amoxicillin Aml<sub>10</sub> µg, and amoxicillin-clavulanic acid AX<sub>25</sub> µg, followed by neomycin N<sub>10</sub> µg (60%), co-trimoxazole COT<sub>25</sub> µg, ceftaxime CX<sub>30</sub> µg (58%), lincomycin-spectinomycin LS<sub>109</sub> µg (45%), cefotaxime clavulanic acid CTC<sub>40</sub> µg (40%), vancomycin VA<sub>30</sub> µg (36%), kanamycin K<sub>30</sub> µg (25%), chloramphenicol C<sub>30</sub> µg (18%), doxycycline DO<sub>5</sub> µg (16%), gentamycin GEN<sub>10</sub> µg (8%).

From the previous results, 49 of MDR strains for the almost tested antibiotics were selected and subjected to further molecular identification to detect the genes oprL of *P. aeruginosa*, ESBL-encoding genes, and carbapenemase-encoding genes (Figure 1).

The molecular detection of *P. aeruginosa* was higher in chicken ( $n = 34$ ) following by Turkey ( $n = 14$ ), whereas the lowest rate found in pigeon ( $n = 1$ ). The molecular investigation of ESBLs producing isolates for (TEM, Ctx-M, and Shv) as presented in Table 7, revealed that the most infected organ in chicken was liver (12 out of 15), heart (3 out of 4), air sac (3 out of 6) whereas the isolates of yolk sac and table egg show 100%. The most prevalent site in turkey was heart and

**Table 7.** Molecular characterization of *P. aeruginosa* and resistant genes.

Source tissue	<i>Pseudomonas aeruginosa</i>			ESBL-encoding genes			Carbapenemase-encoding genes		
	Chicken	Turkey	Pigeon	Chicken	Turkey	Pigeon	Chicken	Turkey	Pigeon
Liver	15	3	1	12	2	0	4	2	0
Heart	4	4	0	3	4	0	0	2	0
Kidney	3	0	0	1	0	0	0	0	0
Air sac	6	2	0	3	0	0	1	0	0
Yolk sac	4	0	0	4	0	0	1	0	0
Subcutaneous tissue swabs	0	4	0	0	4	0	0	1	0
Table egg	2	0	0	2	0	0	0	0	0
Sinus	0	1	0	0	0	0	0	0	0
Total	34	14	1	25	10	0	6	5	0

**Table 8.** Molecular detection of ESBL and carbapenemase-encoding genes.

Encoding genes	Resistant genes	Chicken						Turkey				
		Liver	Heart	Kidney	Air sac	Yolk sac	Table egg	Liver	Heart	Kidney	Air sac	S/C
ESBL-encoding genes	<i>blaTEM</i>	12	3	1	3	3	2	2	4	0	0	4
	<i>blaSHV</i>	4	0	1	0	2	0	2	4	0	0	4
	<i>blaCTXM</i>	9	1	1	3	2	0	2	4	0	0	4
Carbapenemase-encoding genes	<i>blaKPC</i>	1	0	0	1	0	0	1	0	0	0	0
	<i>blaOXA-48</i>	1	0	0	0	0	0	1	0	0	0	0
	<i>blaNDM</i>	4	0	0	1	1	0	2	2	0	0	1

subcutaneous tissue (4 out of 4) followed by liver (2 out of 3). Most of the isolates were found to harbor one or more of the investigated genes as shown in [Table 8](#) where TEM gene was the most prevalent gene with a percentage of 100% at the same time the isolates harbored at least 2 different ESBLs genes.

### Pathogenicity Testing of MDR *P. Aeruginosa*

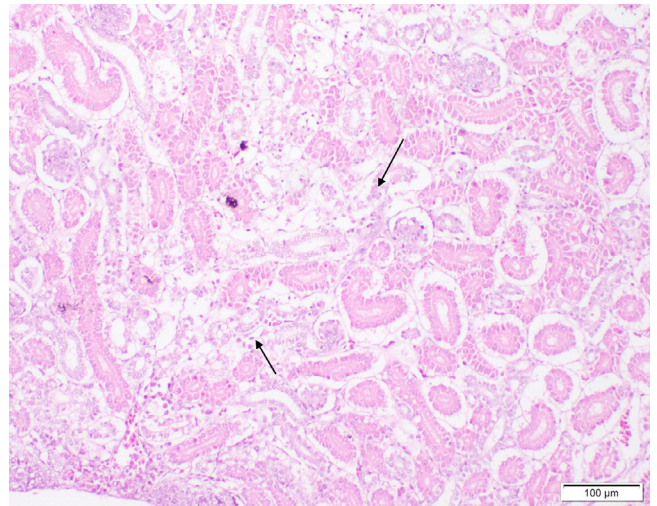
The pathogenicity testing of molecularly identified highly resistant strain revealed 100% morbidity and 100% mortalities in G1 within 48 h post-S/C inoculation of *P. aeruginosa* and the birds in this group showed a septicemic picture in postmortem (PM) examination appeared in the shape of congestion of subcutaneous tissue and muscled, engorgement of subcutaneous blood vessels, and enlargement with congestion in the parenchymatous organs while G2 revealed 80% morbidity and 40% mortality within week postoral infection with highly resistant strain of *P. aeruginosa* and challenged birds in this group showed clinical signs of depression, off food, huddled together, ruffled feathers, brownish mixed with white color diarrhea, some birds showed hard respiration and the PM lesions in this group were nephritis with distended ureters with ureates, congestion with subcapsular hemorrhages in liver with distended gall bladder, air sacculitis, unabsorbed yolk sac, pericarditis, enteritis, and enlarged congested spleen. On the hand, G3 showed neither clinical sign nor mortalities during the observation period.

For reisolation, the bacteriological examination of the tissue samples (liver and heart) which were collected from freshly dead birds showed suspected colonies for *P. aeruginosa* on Cetrimide agar, *Pseudomonas* agar, and MacConkey agar. No organism was isolated from the control negative birds in Group 3.

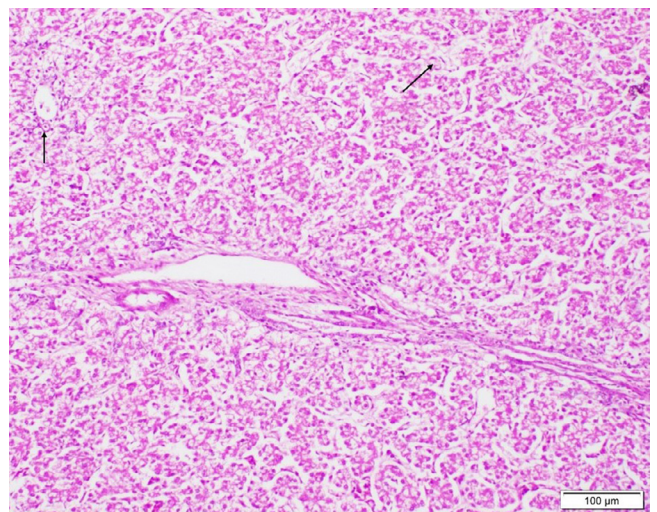
### Histopathological Examination

Freshly dead birds in both Groups 1 and 2 revealed different histopathological lesions in different organs, the lesions were mainly of degenerative nature. Necrosis of the epithelial lining of renal tubules in kidney was observed ([Figure 2](#)), we noted multiple foci of hepatocytic coagulative necrosis in liver ([Figure 3](#)) and degenerative changes of heart muscle bundles ([Figure 4](#)). The

inflammatory reaction was noted and manifested by infiltration of inflammatory cells in the renal interstitial tissue ([Figure 2](#)), congestion of heart blood vessels and edema between cardiac muscle bundles were observed as well ([Figure 4](#)).

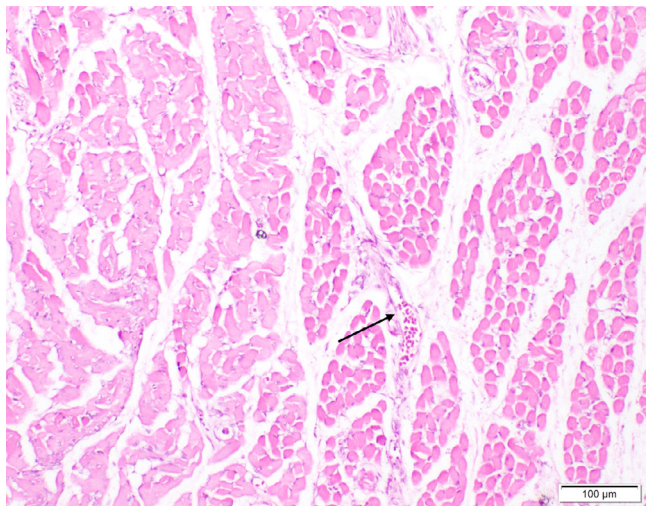


**Figure 2.** Kidney of chicks showing necrobiotic changes of the epithelial lining of renal tubules with focal infiltrations of inflammatory cells (arrows) (H&E, scale bar = 100  $\mu$ m).



**Figure 3.** Liver of chick showing multifocal areas of coagulative necrosis (arrows) (H&E, scale bar = 100  $\mu$ m).





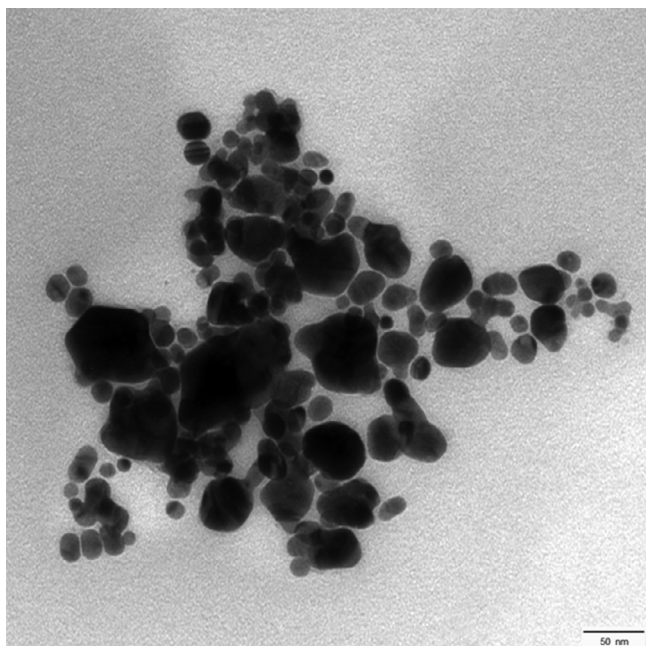
**Figure 4.** Heart of chick showing congestion of blood vessels (arrow), edema between cardiac muscles and degenerative changes of muscle bundles (H&E, scale bar = 100  $\mu$ m).

### Silver Nanoparticles Preparation and Characterization

Transmission electron microscopy (TEM) imaging of the synthesized silver nanoparticles shows their spherical shape. The mean diameter of the nanoparticles was 33 nm (Figure 5).

### In Vitro Assessment of Silver Nanoparticles Efficacy Against Resistant Strains of *P. Aeruginosa*

Minimal inhibitory concentration assay: The bacterial growth of *P. aeruginosa* was completely inhibited after



**Figure 5.** TEM image shows the morphology of silver nanoparticles which appear as spheres with a mean diameter of 33 nm (scale bar = 50 nm).

24 h incubation with silver nanoparticles (25  $\mu$ g/mL) and this was evidenced by the complete absence of bacterial growth on agar plates, but other concentrations (12.5, 6.25, and 3.13  $\mu$ g/mL) showed incomplete inhibition of bacterial growth.

## DISCUSSION

The hatchery is the primary source of disease transmission in the poultry industry, the issue often begins with infected eggs that have been incubated under suitable conditions for microbiological development, from dead in shell embryos, various bacterial pathogens that contaminate hatcheries have been isolated especially *P. aeruginosa* (Shahat et al., 2019; Abd El-Ghany, 2021; Algammal et al., 2023).

In this study, the overall isolation percentage from all examined samples was 36.2% (571/1,576) representing 45.2% (532/1,176) from different birds' tissues and 39/400 (9.7%) from total egg samples. In Egypt, lower isolation rates were reported by Mohamed (2004) who detected *P. aeruginosa* with incidence of 8.7%, and 17.6% from broiler chicken flocks in various governorates. Hassan (2013) recovered *P. aeruginosa* from diseased with isolation percentage of 25.3% and from dead broiler chicks with isolation percentage of 10% also, Eraky et al. (2020) isolated *P. aeruginosa* with incidence of (8%) from hatcheries and 8.25% from dead in shell embryos. Shukla and Mishra (2015) isolated *P. aeruginosa* with incidence of (20%) from 4-days-old sick chicks in Jabalpur. The variations in the isolation rate may be contributed to the differences in the collected samples, isolation media, isolation procedure, and the isolation conditions.

In this study, *P. aeruginosa* was isolated from different organs (heart, liver, kidney, air sac, yolk sac, subcutaneous tissue, external eggshell swabs, and internal egg content) also, Shukla and Mishra (2015) recovered *P. aeruginosa* from heart, lung, liver, and air sacs of 4-day-old broiler chickens.

*Pseudomonas* infection was considered an extensive economic problem in poultry farms, especially *P. aeruginosa* which causes a high mortality in poultry (Elsayed et al., 2016). The complications caused by *P. aeruginosa* in poultry have appeared in the form of respiratory signs, septicemia, keratitis, and sinusitis (Hai-ping, 2009).

In this study, the molecular detection of *P. aeruginosa* was higher in chicken ( $n = 34$ ) following by Turkey ( $n = 14$ ), whereas the lowest rate found in pigeon ( $n = 1$ ). Our findings were consistent with those of Shahat et al. (2019), who isolated *P. aeruginosa* at a 42% incidence from dead broiler chicks. OPRL genes are thought to be a specific marker for molecular detection of *P. aeruginosa* and encode a protein in the inner and outer membranes that is necessary for the invasion of epithelial cells (De Vos et al., 1997). This reflects on the use of OPRL genes in molecular detection which confirmed the existence of *P. aeruginosa*.

However, the results of molecular identification of *P. aeruginosa* from turkey is dramatically different from [Estepa et al. \(2015\)](#) who detected only 5 isolates of *P. aeruginosa* from which one of them only isolated from turkey meat.

The results in [Table 7](#) showed that the most isolated site of *P. aeruginosa* in chicken was liver followed by air sac, yolk sac then heart and kidney. On other hand, [El sadda et al. \(2021\)](#) found the most isolated site was yolk sac 15.5% (13 out of 84), liver 4.5% (9 out of 200); 1.5% (3 out of 200) from heart blood, and zero from kidney, while in turkey the most prevalent organ was heart and subcutaneous tissue; liver and air sac then the lowest isolated organ was sinus.

*P. aeruginosa* gains attention as a dangerous disease for consumer health that causes a variety of infections in people and food animals and possesses features that are transferrable to other pathogens that affect both people and animals ([Wood et al., 2023](#)). One of the biggest issues facing the globe today is antimicrobial resistance, which is getting worse in developing nations ([El-Saadony et al., 2023](#)). As a result, it is critical to diagnose *P. aeruginosa* accurately and rapidly and determine its susceptibility pattern to prevent unnecessary antibiotic use that could result in antibiotic-resistant organisms ([Hamisi et al., 2012](#)).

The molecular investigation of ESBLs producing isolates for (TEM, Ctx-M, and Shv) as shown in [Table 7](#), revealed that the most infected organ in chicken was liver (12 out of 15), heart (3 out of 4), air sac (3 out of 6) whereas the isolates of yolk sac and table egg show 100%. On other hand, the most prevalent site in turkey was heart and subcutaneous tissue (4 out of 4) followed by liver (2 out of 3). Most of the isolates were found to harbor one or more of the investigated genes as shown in [Table 8](#) where at least 2 different ESBL genes were present in the isolates, with 100% prevalence of the TEM gene being the most common gene. The genes for TEM-1, TEM-2, SHV-1, and other  $\beta$ -lactamases are the source of ESBLs due to mutations that change the amino acid structure surrounding their active sites. The range of  $\beta$ -lactam antibiotics susceptible to hydrolysis by these enzymes is thereby expanded ([Paterson and Bonomo, 2005](#)). Plasmids are routinely used to encode the ESBLs whereas genes encoding resistance to different drug classes are typically included in plasmids that produce ESBLs.

Our results are sharply different from [Radwan et al. \(2018\)](#) who confirmed ESBLs phenotypically in 10 (40%) out of 25 isolates of *P. aeruginosa* recovered from inflamed kidneys. From which Ctx-M gene was the most prevalent gene with a percentage of 50%.

Along with table egg, the high prevalence of ESBL-encoding genes (100%) and this can facilitate the spread of multidrug-resistant organisms (MDRO). This results can be is worrying, as these can contribute to not only local but global dissemination of antimicrobial resistance and this agrees by [Dierikx et al. \(2013\)](#). Studies have demonstrated that ESBL producers are persistently carried by chicken ([Huijbers et al., 2016](#)). According to

[Nilsson et al. \(2014\)](#) and [Reich et al. \(2013\)](#), ESBL producers are separated from broiler chickens and grand-parent breeding stock and this ease the transmission to the surrounding environment especially water and soil leading to the potential hazard to the human. At the same manner, eggs considered one of the main food chain, the presence of MDRO on it facilitate its spreading to human consequently being one of the causative agents for infections with limited therapeutic options ([Bettiol and Harbarth, 2015](#)).

Carbapenems are beta-lactam antibiotics that are frequently used as a last-resort antimicrobial against MDRO ([Temkin et al., 2014](#)). Carbapenems are effective against gram-negative bacilli that produce ESBL and AmpC. Because of the widespread spread of MDRO, these antimicrobials have recently become widely used in human medicine. As a result, carbapenem resistance has spread rapidly, and it is now a common occurrence in hospitals and, to a lesser extent, community settings.

The results of carbapenemase-encoding genes as shown in [Table 5](#) illustrated that its rate in chicken is approximately like turkey but differ in the isolation sites where the most prevalent organ in chicken was liver (4 out of 15) then air sac (1 out of 6) and yolk sac (1 out of 4). However, liver and heart were the most prevalent organs in turkey with percentage rate 66% (2 out of 3) and 50% (2 out 4) relatively. Sinus of turkey shows 25% (1 out of 4) isolation rate of carbapenemase-encoding genes. All isolates were carrying NDM type as shown in [Table 6](#) and this agree with [Abdallah et al. \(2015\)](#) who mentioned that the NDM group was found in all carbapenemase-producing Enterobacteriaceae isolates from retail chicken meat in Egypt. [Hamza et al. \(2016\)](#) detected 43% of their isolates from chicken were carbapenem-resistant and all of them were positive for blaNDM.

Since the usage of carbapenem “which are a class of beta-lactam antibiotics that are active against a wide range of organisms” is prohibited in veterinary medicine, its presence indicates the contamination of the chicken farms either from the infected human carrier or sewage effluent which may contaminate the drinking water inside poultry farms and subsequently infect the poultry and residues of resistant genes remain inside different organs causing human health risk in the public sphere.

From our results, the pathogenicity testing of MDR *P. aeruginosa* strain revealed 100% morbidity and 100% mortalities in G1 and the birds in this group showed a septicemic picture in PM examination while, G2 revealed 80% morbidity and 40% mortality within week postoral challenge and the birds showed a variety of clinical signs and PM lesions. Similar findings were reported by [Shukla and Mishra \(2015\)](#) who challenged chicks with *P. aeruginosa* with 2 different methods as intramuscularly (I/M) with 0.25 mL of the broth culture or via swapping the palatine cleft, and reported 100% mortalities in I/M infected birds within 24 h with the reisolation of the organism, while, they recorded 30% mortalities in those infected via the swapping of the palatine cleft within 72 h after challenge and they also,



recorded similar signs and noticed lungs congestion, liver paleness with petechial hemorrhages, impacted gall bladder, enteritis and intestine filled with cheesy sticky substances, distended cloaca, and unabsorbed yolk in PM examination of freshly dead birds.

Bellanti et al. (1987) referred to that one of the adverse reactions of vaccination is the transmission of pathogens especially *Pseudomonas* spp. via contaminated inactivated vaccines. From the results of S/C injection of *P. aeruginosa* and the resulted 100% mortalities, call us to emphasize the need to ensure that inactivated vaccines are free from contamination with the *P. aeruginosa*, and to make sure that sterile syringes are used when dealing with birds to avoid the spread of the *P. aeruginosa* through contaminated inactivated vaccines and/or contaminated syringes, which may in turn lead to 100% mortality as a result of the transmission of *P. aeruginosa* to birds through injections.

Regarding histopathological lesions, the characteristic lesions of *P. aeruginosa* infection were mainly of degenerative and inflammatory nature. Focal hepatic necrosis found in this study was previously reported in *P. aeruginosa* infected chicks (Supartika et al., 2006). Edema found between heart muscle bundles are attributed to the effects of *P. aeruginosa* toxins on the permeability of endothelial junctions (Rejman et al., 2007).

Nanoparticle applications in veterinary medicine are surging (Shaalán et al., 2016; Abdel Ghany et al., 2021; Abu-Elala et al., 2021). Nanoparticles show potential activity against different bacterial species including multidrug-resistant ones (Lang et al., 2021; Elgendy et al., 2022). In fact, Ag-NPs have improved universal antibacterial, antiviral and antiparasitic activities (Rizzello and Pompa, 2014). Specifically, silver nanoparticles are one of the most potent nanoantibacterial agents studied in the literature, they are employed in a variety of avian production processes, including as detergents formulations in hatcheries (Banach et al., 2016), in addition, studies have been applied to show their efficacy against various pathogens (Farouk et al., 2020; El-Adawy et al., 2021).

From our in vitro results, Ag NPs revealed a potent antibacterial activity against MDR *P. aeruginosa*. Our results are parallel to those conducted by El-Adawy et al. (2021) who referred to its antibacterial activity against *P. aeruginosa* strain isolated from fish, Salem et al. (2021) who confirmed the efficacy of Ag-NPs against *Clostridia perfringens* in broiler chickens, Farouk et al. (2020) showed that it was effective against MDR *Salmonella* spp., Vadalasetty et al. (2018) studied its potency against *Campylobacter jejuni* in chickens and Attia et al. (2022) who confirmed the antiparasitic efficacy of chitosan-silver nanoparticles against *Pseudolychnia canariensis* in pigeons.

The antibacterial activity of Ag-NPs could be attributed to its ability to disrupt and penetrating the microorganism's cell membrane, resulting in damage to the cell wall and cytoplasm leakage, and through their intimate association with the thiol groups found in the primary respiratory enzymes, Ag NPs have been

demonstrated to have antibacterial inhibitory activities (Gordon et al., 2010; Hassan et al., 2021).

## CONCLUSIONS

Multidrug-resistant *P. aeruginosa* hazard threatens the poultry industry revealing high morbidity and mortalities at first week of age with a zoonotic hazard. In this study, some strains showed MDR against (kanamycin, amoxicillin, amoxicillin-clavulanic, neomycin, chloramphenicol, vancomycin, cefotaxime clavulanic acid, lincomycin-spectinomycin, co-trimoxazole, ceftiofur, gentamycin, and doxycycline) which were confirmed by the molecularly detected (ESBL and carbapenemase-encoding genes). The selected MDR strain showed severe pathogenicity with histopathological tissue change in both S/C and oral challenged birds inducing 100 and 40% mortalities, respectively. MDR *P. aeruginosa* strain sowed in vitro sensitivity to silver nanoparticles, and further studies are recommended for the in vivo evaluation of the effect of different concentration of silver nanoparticles against *P. aeruginosa* experimental infection with studying its residual effect in the edible part of chicken meats. The control of *P. aeruginosa* and overcoming MDR requires effective alternative treatments to antibiotics side by side with the application of hygienic measures in both hatcheries and poultry farms levels.

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## DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in the present study.

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