Iraqi Journal of Veterinary Sciences

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Effect of garlic oil nanoemulsion against multidrug resistant *Pseudomonas aeruginosa* isolated from broiler

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Article information

Article history:
Received December 10, 2021
Accepted April 6, 2022
Available online September 4, 2022

Keywords:
Resistance
Pseudomonas spp.
Garlic oil
Virulence gene

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Abstract

This work aimed to focus on the antibacterial properties of garlic nanoemulsion on some multidrug resistance (MDR) strains of *Pseudomonas aeruginosa* isolated from broiler farms and hatcheries in Sharkia and Ismailia governorates, Egypt. *Pseudomonas* spp. was isolated in 21.3% of collected samples. It was isolated from younger broilers 1-10 days with an incidence rate of 22% (11/50), older broilers 16% (8/50), dead embryo in shell 31.4% (11/35), and from hatcheries was 13.3% (2/15). There was a variable range of antibiotic resistance ranging from 66.7-100% against the isolated strains of *P. aeruginosa*. Tetracycline and sulfamethoxazole-trimethoprim achieved the highest resistance rates, while penicillin and gentamycin were of a lower rate. However, norfloxacin, ciprofloxacin, and colistin were the most sensitive antibiotics against examined MDR *P. aeruginosa*.16SrDNA gene was found in ten *P. aeruginosa* isolates. These isolates were found to be virulent as *oprL* gene was detected in all isolates 100%. In addition, *tetA(A)*, *bla*TEM, *arr*, and *mex*R antibiotic resistance genes were shown positive 100% in all MDR *P. aeruginosa* isolates. Minimum Inhibitory Concentration (MIC) values showed that garlic nanoemulsion (GN) was effective against examined *P. aeruginosa* at different concentrations. GN had 29.61% sulfur compounds of active components with 0.52 ug/ml of IC_{50} and 40.94 nm size with polydispersity index: 0.165 using dynamic light scattering had a 19.6± 5.11mV. In conclusion, the application of garlic nanoemulsion is an excellent alternative candidate to antibiotics for treatment because it significantly reduced the gene expression levels of MDR *P. aeruginosa* in broiler farms.

DOI: 10.33899/IJVS.2022.132430.2094,

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes severe bacterial respiratory or septicemic problems in chicken farms (1). The mortality rate of newly hatched chicks was high in the later stages due to *P. aeruginosa* infection. (2). Various bacterial pathogens were isolated from dead embryos inside the eggs (3). The problem constantly begins when the incubated eggs have been contaminated with the organism from the surrounding environment (4). The multifactorial infection process of *Pseudomonas* is referred to their possession of several virulence determinants, including either cell-associated or extracellular factors such as lipopolysaccharide, alkaline protease, elastase, hemolysins, phospholipase "C" rhamnolipids, biofilm, Pilli, and flagella that induce its toxicity and pathogenicity (5,6). The mechanism of yolk sac infection with *Pseudomonas spp.;* it degrades yolk proteins causing infection since it is extensively colonized, producing more tissue damage than they could invade the blood,

causing septicemia and significant mortalities in chickens (7). Biofilm formation, ability to induce chronic infections, the opportunistic nature of *P. aeruginosa*, and other factors is accounted for the high level of multiple drug resistance of this species (8). Recent studies confirmed multiple drug resistance *P. aeruginosa* against many antibiotics (9,10). Therefore, the WHO organization has recently proclaimed its extreme demand to develop new antibiotics to treat infections with MDR *Pseudomonas spp* (11). The evolution of multi-resistance problems against different classes of antibiotics and consequently the emergence of drug-resistant strains paid attention to nanomaterials (12). Nanomaterials are defined broadly as ecologically materials that have been utilized in numerous assortments of applications (12). Previous studies showed the mechanisms of a nanomaterial as antibacterial through different mechanisms. For example, bacterial cell division interacts, specifically, biofilm arrangement hindrance, enactment of both natural and versatile host immune response, the era of reactive oxygen species (ROS), and DNA or proteins interaction as intracellular induction effects (13). Furthermore, these materials can disturb the bacterial film acting on intracellular components, causing the malfunction of the cellular machinery (13). The high significant value of nanotechnology is stated due to the ability to manipulate, characterize, fabricate materials or devices, which have different dimensions (14). Nanoemulsions typically possess dimensions below 500 nm and have enhanced assimilation properties achieved through the mucosa (15). It demonstrates a high level of suspension stability and is produced through high-energy or low-energy methods due to their highly reduced dimensions (16).

Recent research is lacking specifically for garlic oil nano-emulsification and its relevant properties. For this reason, this study was aimed to create nano-emulsified garlic oil, investigate the antibacterial properties of some virulent MDR strains of *P. aeruginosa* in broilers and discuss their in-vitro genetic expression after treatment with Nano-garlic.

Materials and methods

Ethical statement

The Animal Health Research Institute's (AHRI) Research Ethics Committee for Environmental and Clinical Studies approved the animal studies, which were carried out in accordance with the Egyptian Ethics Committee's guidelines and the National Institutes of Health's (NIH) Guidelines for the Care and Use of Laboratory Animals. The protocol number for these studies was 165590.

Sample collection

About 150 samples were aseptically collected from different broiler farms at Sharkia and Ismailia governorates, Egypt. Fifty samples of chicks (1-10 days old) and 50 cases of broilers in older ages. The represented samples for each case were included liver, heart, and lung samples. Moreover, 35 swabs from yolk sacs of late dead in shell embryo and 15 swabs from hatcheries were also collected. All samples were kept in the icebox and transported to the laboratory for bacterial isolation of *Pseudomonas* spp.

Bacterial isolation and identification

The samples were cultivated and enriched onto 9 ml nutrient broth and incubated aerobically at 37°C for 24 h. The cultivated broth was sub-cultured onto selective *Pseudomonas* F-agar for specific identification of *Pseudomonas* spp and on MacConkey agar to detect the non-lactose fermenting activity and onto nutrient agar plates "to observe the characteristic pigmentation of *Pseudomonas* spp. Then, the plates were incubated aerobically at 37°C for 24 h. The suspected colonies were picked up and further identified based on colonial morphology, detection of smelly scent, hemolytic activity, sugar fermentation, and biochemical features (17). The pure isolates of *Pseudomonas spp* were transferred to 1% nutrient agar slant and stored at 4°C for serology and PCR

Serological identification

All the recovered isolates of *Pseudomonas spp* were subjected to slide agglutination test (polyvalent and monovalent *P. aeruginosa* antisera) for specific detection of somatic antigen (O) ". The test was carried out in Animal Health Research Institute, Dokki, Egypt using antisera from Denka Seiken Co. Ltd, Tokyo, Japan. Positive slide agglutination indicates a positive reaction of agglutination (18).

Antibiotic susceptibility testing

The most identified *P. aeruginosa* isolates were screened against nine commercial antimicrobial agents: streptomycin 10 µg, gentamycin 10 µg, tetracycline 30 µg, doxycycline 30 µg, penicillin 10 µg, colistin 10 µg, norfloxacin 10 µg, ciprofloxacin 5 µg and sulfamethoxazole-trimethoprim 25 µg with standard disc diffusion method and the results were interpreted as recommended by CLSI (19). The susceptibility of identified isolates resistant to three or more antibiotics was classified as multidrug drug resistance (MDR) strains.

DNA extraction and PCR amplification

DNA was extracted according to QIAamp DNA mini kit instructions from isolates, details oligonucleotide primer supplied from Metabion (Germany). PCR reaction 25 µl contained 12.5 µl of EmeraldAmp GT PCR Master Mix (Takara, Japan), one µl of 20 pmol concentration of each primer, 4.5 µl of water, and six µl of the DNA template. PCR reactions were performed in Applied Biosystems 2720 Thermal Cycler. Each PCR product was loaded in a separate

well in 1.5% agarose gel, then photographed and analyzed using a gel documentation system (Alpha Innotech, Biometra, Germany) through its computer software (Tables 1 and 2).

Table 1: Primers used for the amplification and Sequences of different genes in *P. aeruginosa* isolates

Target gene	Function of target gene	Primers sequences	Size (bp)
*16S r*DNA	Conserved gene	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	618
*opr*L	Outer membrane lipoprotein virulence gene	ATGGAAATGCTGAAATTCGGC CTTCTTCAGCTCGACGCGACG	504
*bla*TEM	Beta lactamases resistance gene	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516
*tet*A(A)	Tetracycline resistance gene	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576
arr	Aminoglycoside response regulator gene	AGCGCATCACCCCCAGCAAC CGCCAAGTGCGAGCCACTGA	686
*mex*R	Multidrug resistance gene	GCGCCATGGCCCATATTCAG GGCATTCGCCAGTAAGCGG	637

Table 2: Cycling conditions used for the amplification of different genes in *P. aeruginosa* isolates

Target gene	Size (bp)	Denaturation ºC/min	Amplification (35 cycles) ºC/sec			Final extension ºC/min
			Denaturation	Annulation	extension	
16Sr DNA	618	95/5	94/30	50/40	72/45	72/10
*opr*L	504	95/5		55/40		
*bla*TEM	516	95/5		54/40		
*tet*A(A)	576	95/5		50/40		
arr	686	94/5		55/40		
*mex*R	637	94/5		55/40		

Preparation of nano garlic emulsion

The materials used in this study: Garlic oil; was purchased from oils extract a unit of National Research Center (NRC), Tween80; was obtained from Sigma-Aldrich Co. and deionized water. The garlic oil and tween 80 were mixed with a homogenous blender (1000 watts) for 5 min, then distilled water was added slowly to the mixed oil phase. The concentration of garlic oil micro-emulsion was 20% oil in water. Nano garlic emulsion was performed in Nanomaterials Research and synthesis unit (20).

Characterization of garlic oil nanoemulsion

Nanoemulsion was done using Fourier transmittance High-resolution transmission electron microscopy (HRTEM), then the results were observed via a JEM 1400F HRTEM at a beam energy of 300 keV. The components of garlic nanoemulsion using GC-MS at Nawah Scientific Inc. (Mokatam, Cairo, Egypt) and Zetasizer Malvern Instrument (Corp, Malvern, UK) measured the electrical conductivity, surface charge (zeta potential), droplet size, and size distribution (polydispersity indexes PDI) of the nanoemulsion material.

Cell culture

In this study, Vero (or green monkey) cell line was obtained from Nawah Scientific Inc. (Mokatam, Cairo, Egypt). The cells were maintained in DMEM media, supplemented with 100 mg/mL of streptomycin (100 units/mL) of penicillin and 10% of heat-inactivated fetal bovine serum in a humidified atmosphere with 5% CO_2 (v/v) at 37ºC.

Cytotoxicity assay

The cell viability was assessed by SRB (sulforhodamine B) assay with different concentrations 0.01, 0.1, 1, 10 and 100 ug/ml (21).

Estimation of MIC of nano-garlic

Minimum Inhibitory Concentration (MIC) was estimated using 96 well-plates to detect the antibacterial effect of garlic nanoemulsion against ten *P. aeruginosa* isolates. First, 50 ul of peptone water broth was dispensed in each well of the column, then 50 ul of the garlic nanoemulsion was added in column "1". Double serial dilutions were performed using a multichannel pipette for transferring and mixing garlic nanoemulsion from column 1-10. About 50 ul of *P.*

aeruginosa broth (1.5 x10^5 CFU /ml) was dispensed in each well of the column. Then they were incubated for 24 h at 37 °C. After incubation, 30 ul of 0.015% of resazurin was added and re-incubated for 2-4 h (for the observation of any color change). Columns with no change in color (blue resazurin color remained unchanged) were scored above the MIC value (22,23). Raw 1-10 served as ten *P. aeruginosa* isolates, column 1-10 two-fold serial diluted garlic nanoemulsion, column 11 negative control, and column 12 *P. aeruginosa* isolates positive control.

RNA extraction

first, before RNA purification from bacterial harvests, 0.5 ml of the fresh bacterial broth was mixed with 1 ml of RNA protect bacteria reagent (Qiagen, Germany, GmbH). To prevent bacterial RNA degradation, keeping the components for 5 min at room temperature; 200 µl of Tris EDTA buffer containing 1 mg/ml lysozyme (Thermo Fisher Scientific, GmbH, Germany) was added the pelleted bacteria. Bacterial RNA extraction was performed according to the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). During RNA extraction, on-column DNase digestion was done to remove residual DNA.

SYBR green Rt-PCR

PCR reaction was applied in Stratagene MX3005P real-time PCR machine using specific primers as listed in table 2. Specific primers were utilized in a one-step 25 µl reaction comprising 12.5 µl of the 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of RevertAid Reverse Transcriptase (200 U/µL) (Thermo Fisher Scientific, GmbH, Germany), 0.5 µl of different primer (20 pmol conc.), 8.25 µl of PCR grade water, and 3 µl of purified RNA.

Data analysis of the SYBR green rt-PCR

The relative expression of each resistant gene was normalized using the related bacterial housekeeping gene. Relative quantitation of gene expression on the RNA templates of the different samples was estimated using an untreated control sample to compare the CT value of each sample through the ΔΔCt method. The samples were tested in triplicates (24).

Results

Bacterial isolation, cultural characteristics and biochemical identification

According to morphological and cultural characteristics, the recovered isolates were of *Pseudomonas spp.* They exhibited a characteristic green, bluish color with *Pseudomonas* F-agar medium's fruity odor. They were non lactose fermenters on MacConkey agar medium. Biochemically, they gave positive reactions for oxidase, catalase, urea, citrate utilization, and gelatin hydrolysis tests, but indole, methyl red, and Voges Proskauer tests were adverse reactions. They ferment glucose, mannose, and xylose sugars. However, they were: sucrose, lactose, and maltose negative. On triple sugar iron (TSI) agar medium, *P. aeruginosa* produced red butt and slant without H_2S production.

The overall prevalence ratio of *Pseudomonas* spp. in all examined samples in broiler of different ages and from hatcheries was 21.3% (32/150) (Table 3). A higher percentage of *Pseudomonas spp.* was detected in the young age of broiler chicks (1-10 days) (11/50) 22% than older ages of broilers (8/50) 16%. Moreover, it was recorded in 11 of 35 from late dead in shell embryo, but it was isolated in 2 of 15 samples from the hatcheries.

Serological typing recovered in *Pseudomonas* spp. isolates confirmed that 32 isolates belonged to *Pseudomonas* spp. except only three isolates were serologically negative. The serologically identified isolates were 15 *P. aeruginosa*, nine *P. fluorescens*, *P. putida* (3 isolates), and *P. fragi* (2 isolates). In addition, according to the results of the slide agglutination test, the most prevalent *P. aeruginosa* serotypes were: O_1, O_3, O_6, O_{10}, and O_{11} (Table 4).

Table 3: Prevalence rate of the isolated *Pseudomonas spp*. in chickens

Type of sample	Source	No.	No. of positive isolates	%
Dead in-shell chicken embryos	Sharkia	20	11/35	31.4%
	Ismailia	15		
Young chicks (1-10 days)	Sharkia	25	11/50	22%
	Ismailia	25		
Broilers (over ten days)	Sharkia	35	8/50	16%
	Ismailia	15		
Hatcheries	Sharkia	10	2/15	13.3%
	Ismailia	5		
Total	--	150	32/150	21.3%

Table 4: Serogrouping of identified *P. aeruginosa*

Serotype	Serogroup	Identified isolates (n)
P. aeruginosa O1	M	3
P. aeruginosa O3	G	2
P. aeruginosa O6	G	5
P. aeruginosa O10	A	1
P. aeruginosa O11	G	4
Total		15

Moreover, most of the 15 examined isolates of *P. aeruginosa* exhibited a multi-resistance drug phenomenon. They showed a variable range of resistance rate from 66.7%-100%. The isolates were 100% and 86.7% resistant to tetracycline and sulfamethoxazole-trimethoprim, respectively. However, a moderate rate of resistance was recorded against penicillin and gentamycin in 10 of 15 (66.7%). At the same time, streptomycin and doxycycline showed resistance in 11 of 15 (73.3%). However, ciprofloxacin, colistin, and norfloxacin were highly sensitive in 3/15 (20%), 2/15 (13.3%), and 4/15 (26.7%), respectively, as shown in table 5.

PCR investigation of genotypic virulence attributes of the recovered isolates

Ten examined *P. aeruginosa* isolates with multidrug resistance phenotypic attributes were randomly selected and tested by PCR (Tables 6 and 7). The results revealed that all 10/10 (100%) of the tested *P. aeruginosa* isolates were positive for (*16sr*DNA and *opr*L) genes as demonstrated in (Figures 1 and 2).

Genotypic resistance of the recovered isolates by PCR

Ten examined MDR *P. aeruginosa* isolates were screened for tetracycline and beta-lactamases resistance genotypic attributes (*tet*A A and *bla*TEM), which were confirmed in 10/10 (100%) (Figures 3 and 4). Both aminoglycoside response regulator genes *(arr)* and multidrug resistance gene (*mex*R) also were detected in 100% of *P. aeruginosa* isolates (Figures 5 and 6). These genes were used to evaluate the garlic oil nanoemulsion effect.

Table 5: Phenotypic resistance profile of the examined *P. aeruginosa* strains

Chemotherapeutic group	Chemotherapeutic Agents (dose)	No. of resistant Strains (%)
Aminoglycosides	Streptomycin (10 µg)	11/15 (73.3%)
	Gentamycin (10 µg)	10/15 (66.7%)
Tetracyclines	Tetracycline (30 µg)	15/15 (100%)
	Doxycycline (30 µg)	11/15 (73.3%)
β-Lactams	Penicillin (10 µg)	10/15 (66.7%)
Polymyxins	Colistin (10 µg)	2/15 (13.3%)
Fluoroquinolones	Norfloxacin (10 µg)	4/15 (26.7%)
	Ciprofoxacine (5 µg)	3/15 (20%)
Diaminopyrimidine	Sulfamethoxazole trimethoprim (25 µg)	13/15 (86.7%)

Table 6: Resistance profiles of different virulence and resistant genes of *P. aeruginosa* isolates

No.	Source of isolates	Serotype	Resistant profile
1	Young chicks (1-10 days)	O1	S, T, P, CIP, CT, NOR, DO, CN, SXT
2	Dead in-shell chicken embryos	O3	S, T, P, CIP, CT, NOR DO, CN, SXT
3	Broilers (over than ten days)	O6	S, T, P, CIP, NOR, DO, CN, SXT
4	Hatcheries	O11	S, T, P, NOR, DO, CN, SXT
5	Young chicks (1-10 days)	O10	S, T, P, DO, CN, SXT
6	Dead in-shell chicken embryos	O1	S, T, P, DO, CN, SXT
7	Broilers (over than ten days)	O6	S, T, P, DO, CN, SXT
8	Hatcheries	O11	S, T, P, DO, CN, SXT
9	Young chicks (1-10 days)	O11	S, T, P, DO, CN, SXT
10	Dead in-shell chicken embryos	O6	S, T, P, DO, CN, SXT

S: Streptomycin, T: Tetracycline, P: Penicillin, CIP: Ciprofloxacin, NOR: Norflaxacine, CT: Colistin, DO: Doxycycline, CN: Gentamycin, SXT: Sulfamethoxazole trimethoprim

Table 7: Genotypic characterization of different virulence and resistant genes of *P. aeruginosa* isolates

Isolates no.	Conserved gene	Virulence genes	Resistant genes			
	*16sr*DNA	*opr*L	*bla*TEM	*arr*	*mex*R	*tet*A(A)
1	+	+	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	+	+	+	+	+
6	+	+	+	+	+	+
7	+	+	+	+	+	+
8	+	+	+	+	+	+
9	+	+	+	+	+	+
10	+	+	+	+	+	+
Total (%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)

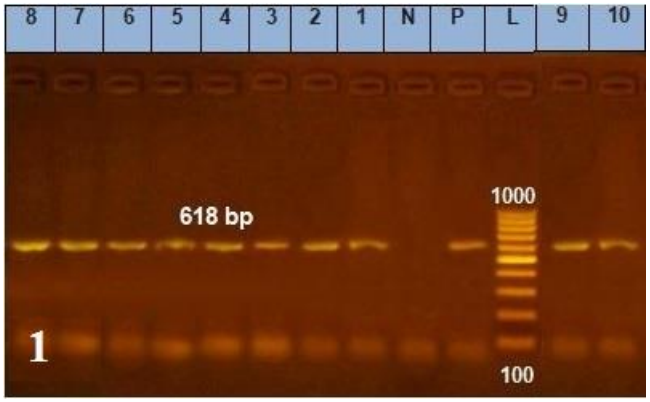

Figure 1: *16sr*DNA gene results at 618bp for polymerase chain reaction products, lane 1-10 positive for, Lane (L): DNA molecular size marker, lane (P): positive control, lane (N): negative control.

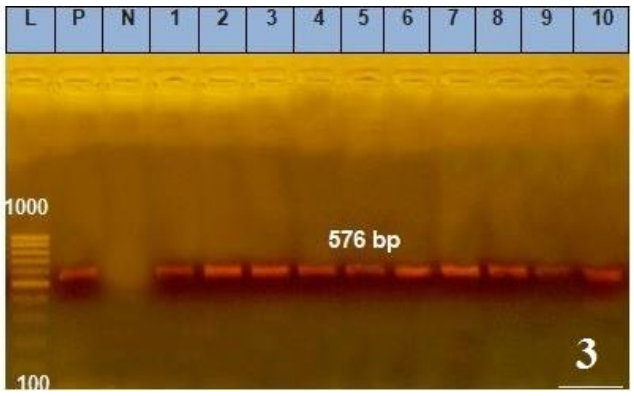

Figure 3: *tet*A(A) gene results at 576bp for polymerase chain reaction products, lane 1-10 favorable, Lane (L): DNA molecular size marker, lane (P): positive control, lane (N): negative control.

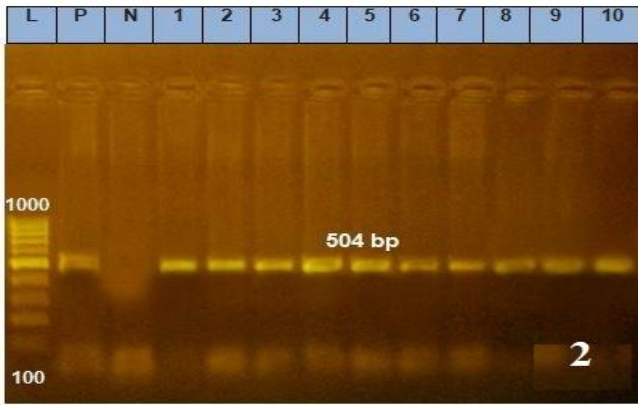

Figure 2: *opr*L gene results at 504 bp for polymerase chain reaction products, lane 1-10 favorable, Lane (L): DNA molecular size marker, lane (P): positive control, lane (N): negative control.

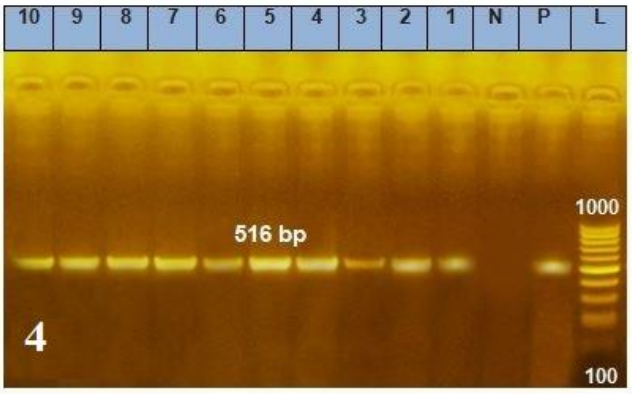

Figure 4: *bla*TEM gene results at 516bp for polymerase chain reaction products, lane 1-10 positive, Lane (L): DNA molecular size marker, lane (P): positive control, lane (N): negative control.

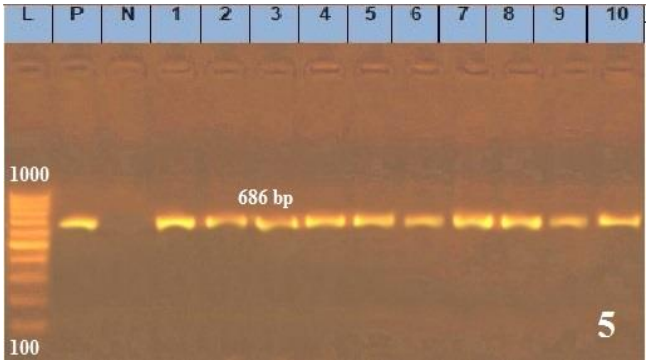

Figure 5: *arr* gene results at 686 bp for polymerase chain reaction products, lane 1-10 positive, Lane (L): DNA molecular size marker, lane (P): positive control, lane (N): negative control.

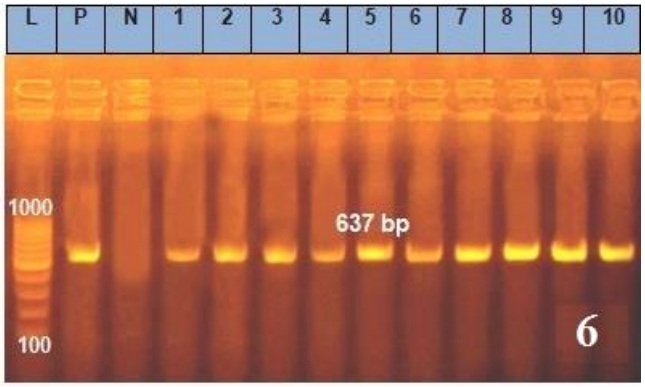

Figure 6: *mex*R gene results at 637bp for polymerase chain reaction products, lane 1-10 positive, Lane (L): DNA molecular size marker, lane (P): positive control, lane (N): negative control.

Characterization of garlic nanoemulsion

Garlic oil nanoemulsion was mainly characterized by TEM non-emulsion size, 40.94 nm with a narrow size distribution (polydispersity index: 0.165), indicating greater homogeneity in nanodroplet size (Figure 7). The zeta potential indicates stable suspensions, generally taken by using dynamic light scattering (DLS), which had a 19.6± 5.11mV, same viscosity 0.08872 (cp), and conductivity 0.36±2.34 ms/cm.

When GC-Mass was analyzed the garlic nanoemulsion, many active components were found relative to sulfur compounds: Trisulfide, dipropyl 2.68%, Tetrasulfide, di-2-propenyl 1.44%, Diallyl disulfide 2.25%, Disulfide, dipropyl 1.28%, Trisulfide, methyl propyl 2.21%, Trisulfide, methyl 2-propenyl 4.37%, Trisulfide, di-2-propenyl 8.00%, Allyl-3-propyl tri sulfane 5.69% and Tetrasulfide dipropyl 1.39%. Other active compounds were also found such as isochiapin B 3.21%, Hexadecanoic acid 4.54%, vaccenic acid 13.41%, N-Methyl-N-benzyltetradecanamine 2.53%, 3-(Benzylmethylamino)-1-propanol 4.28%, 1-Dodecanamine, N, N-dimethyl- 2.32%, 4-dimethylaminoaniline 1.44%, Benzyl chloride 7.74% and Tetrangulol 2.21% as shown in (Figure 8).

On the confluent surface of Vero cells, the obtained results for garlic oil nanoemulsion with different concentrations 0.01, 0.1, 1, 10 and 100 ug/ml three days post inoculation showed that the cell viability% using SRB assay was 97.18, 86.22, 33.69, 6.20, and 0.18%, respectively and IC_{50}= 0.52 ug/ml as shown in (Figure 9).

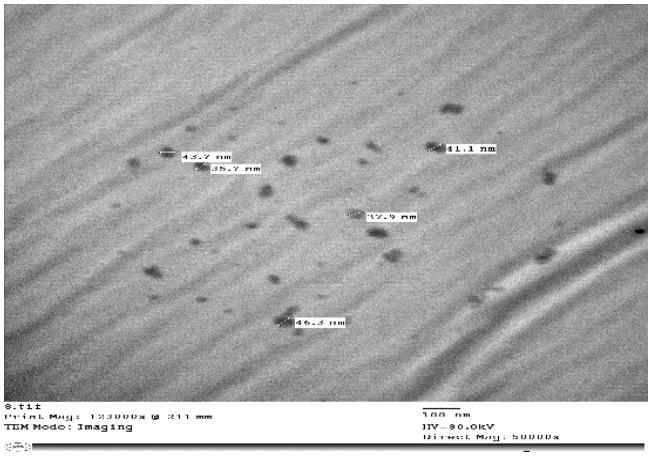

Figure 7: garlic nanoemulsion under HRTEM shown that nano-droplet size, 40.94 nm with a ng greater homogeneity.

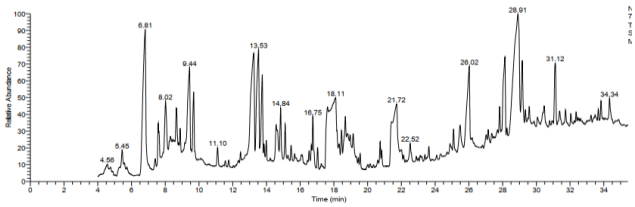

Figure 8: Chemical compounds analysis of garlic nanoemulsion using GC-Mass.

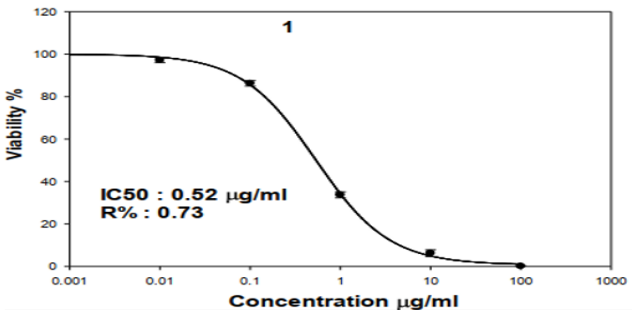

Figure 9: Cell viability of garlic nanoemulsion effect on Vero cells.

Determination of MIC for garlic nanoemulsion against the ten *P. aeruginosa* isolates

The antimicrobial activity and microdilution susceptibility test of garlic nanoemulsion was determined using MIC value as the lowest concentration changed the color (positive result when color turned from purple to pink or colorlessness) against ten examined MDR *P. aeruginosa* isolates. The plates in a *modified* resazurin assay appeared after 24 hours (as pink color indicated growth, but blue color means inhibition of the growth). In this study, column (no. 11) was considered as the negative control (stain and medium), and column (no. 12) was the positive one; (it means a change of resazurin natural color (blue/purple) to the reduced form (red-colorless). The effect of two-fold serial dilation of garlic nanoemulsion (MIC) on ten *P. aeruginosa* isolates revealed that the concentration 1:8 corresponds to the MIC values of isolates (no. 1, 4, 5, 6, 7, and 8) and 1:4 corresponds to isolates (no. 2 and 3), respectively. The concentration effect of isolates no.9 and 10 is 1:16 and 1:32, respectively. Garlic nanoemulsion revealed significant effects and proved that garlic nanoemulsion could stop the growth of *P. aeruginosa* isolates.

Determination of resistant genes expression by real-time PCR

Genes expressions of *mex*R, *arr, tet*A(A), and *bla*TEM genes on the RNA level of five selected MDR *P. aeruginosa* were investigated. Resistant genes expression revealed relatively different degrees of resistant genes downregulation on microbial RNA by real-time PCR concerning the untreated str that ranged from 0.23 to 0.41 for *bla*TEM gene, 0.30 to 0.43 for tetA(A) gene, 0.19 to 0.33 for *arr* gene and 0.16 to 0.24 for *mex*R gene (Figure10).

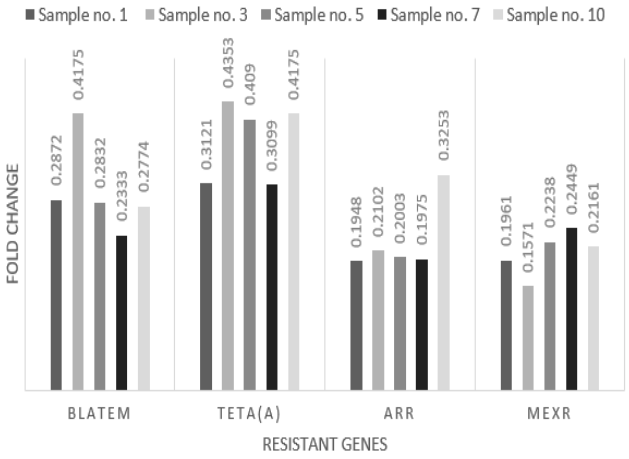

Figure 10: Effect of garlic oil nanoemulsion on the resistance genes expression of *P. aeruginosa* isolates.

Discussion

Pseudomonas aeruginosa is a severe poultry pathogen and an acute hatchery-born disease due to environmental contamination, leading to a severe problem in the poultry industry. Its epidemics may spread rapidly through poultry flocks causing mortality of all ages (25-30). The prescriptive morphological and cultural characteristics of the isolated species in this study revealed Gram-negative bacilli microscopically with typical bluish-green color on *Pseudomonas* agar. At the same time, they were pale on MacConkey agar media, confirming that they were related to *Pseudomonas spp*. The same morphological and cultural features of *Pseudomonas* isolates (4,31).

In this study, the total incidence ratio of *Pseudomonas* spp in broiler farms from chicken and hatcheries was 21.3% (32/150). Similarly, *P. aeruginosa* was yielded in broilers farms in 21, 17.6, 18.6, 19 and 20%, respectively (30-37). Isolates of the Pseudomonas species, including 23 (46%) for beef meat, 11 (22%) for mutton, and 19 (38%) for chicken meat, were obtained from all types of meat 35.33% which identified molecularly by detected of the 16S rRNA gene and rpoB gene (33).

In comparison, a higher percentage 52% was detected by Elsayed *et al.* (2), but a lower rate of *P. aeruginosa* (8%) was also recorded by Betty *et al.* (35) from the diseased chicken with respiratory symptoms. Moreover, 17/372 isolates of *P. aeruginosa* 4.57% from apparently healthy, diseased, and freshly dead chickens were obtained (36). Variations in isolation percentages might be referred to by many factors, including the type of the examined samples, immune status of the bird, degree of contamination, type, and virulence of the strain (37).

On the other hand, recent studies stated that *P. aeruginosa* was detected in young chickens with high mortalities and late embryos dead inside eggs (38-42). In this study, a strong correlation between age and the incidence of *P. aeruginosa* in broilers was observed. A higher isolation rate was detected in age 1-10 days at a rate of 22% (11/50) than older ages of broilers 16% (8/50).

Moreover, the present study shows that *P. aeruginosa* yielded 31.4% (11/35) of late dead in shell embryo and the hatcheries in the percentage of 13.3% (2/15). In the same way, *P. aeruginosa* was found in 19% of unhatched eggs (30). In addition, Balasubramanian *et al.* (43) detected *P. aeruginosa* organisms in 20% of a total of 200 samples of chicks (4 days old age). Results stated that *P. aeruginosa* was recorded in 20% in dead embryos and in19% in broiler chicks (1-10 days) (4). A high incidence of *P. aeruginosa* in dead embryos high mortalities in unhatched chicken and young chicks may occur due to environmental contamination during the hatching time, invasion of eggshell, or insufficient sanitization of hatcheries and incubators since *P. aeruginosa* were ordinarily found in soil, water, and muggy

environments causing hatchery borne diseases in chicken farms (3).

Serological identification of *Pseudomonas spp* is exceptionally imperative since it encourages telling us about the predominant serotypes and finding sources of infection (39). In the current study, the most prevalent species was *P. aeruginosa* (15/32), which were serotyped as O1, O2, O6, O10, and O11. The previous data of *P. aeruginosa* serotyping were achieved with Nashwa *et al.* (40), that the serogrouping of these isolates indicated that the isolates were of A, G, and M serogroups. This result was accomplished with previous studies that illuminated that A, B, D, F, H, K, L, and M were the most predominant serotypes (41).

Multidrug-resistant bacteria are a nowadays authentic hazard in human and veterinary medicine (42). Many researchers studied the susceptibility of *P. aeruginosa* to various antimicrobials, which make it a very hard pathogen to eliminate, and they attributed that *the P. aeruginosa* genome possesses the most prominent known resistance island genes (43).

Moreover, most of the examined isolates of *P. aeruginosa* exhibited a multi-resistance drug phenomenon. In vitro, variable resistance rates 60-100% were recorded in this study. The isolates were 100% and 86.7% resistant to tetracycline and sulfamethoxazole-trimethoprim, respectively. However, a moderate rate of resistance of *P. aeruginosa* isolates was recorded against penicillin and streptomycin: 66.7% (10/15) and 73.3% (11/15), respectively. Meanwhile, they were susceptible to ciprofloxacin 3/15 (20%), colistin 2/15 (13.3%), and norfloxacin 4/15 (26.7%). These results were nearly agreed with Ashraf *et al.* (36), who reported that all *P. aeruginosa* isolates were resistant to tetracycline 88.2%, streptomycin 82.4%, penicillin (76.5%), doxycycline 75.2%, and gentamicin 73.3%, also our results go hand to hand with Shahat *et al.* (4) who obtained high sensitivity of all *P. aeruginosa* isolates with ciprofloxacin and norfloxacin. On the contrary, a lower sensitivity against ciprofloxacin and norfloxacin was illustrated (44).

Variation of our results with previous studies could be referred to the distinction in numerous conditions encompassing incubators or as a result of frequently occurring hyper-mutation among *P. aeruginosa* strains developing various antimicrobial resistance (45). Moreover, antibiotic-resistant bacteria (ARB) can quickly spread alongside the food chain and cause most public health hazards (46,47).

In the current study, which agreed with Shahat *et al.* (4) that *16S rDNA* gene found in *P. aeruginosa* with a prevalence rate of 100%. *P. aeruginosa* is various extracellular virulence factors and cellular components which are implicated in the pathogenesis of this pathogen (48), bacteria could acquire virulence factors from the surrounding environment resulting in cellular damages (49). *opr*L gene is essential for the integrity of *P. aeruginosa* and effluxes transport systems, which affects cell membrane permeability, giving its fundamental reason for antimicrobial resistance in this species (50). This study found the *opr*L gene 100% in all *P. aeruginosa* isolates. The same result was obtained from chicken embryos and broilers isolates (4,25,50).

PCR technique is also applied for studying the antimicrobial genotypic attributes of bacterial isolates by detecting resistant genes. In the current study, PCR confirmed 100% presence of *tet*A (A) and *bla*TEM (for tetracycline and beta-lactamases) antibiotic resistance genes in all ten examined *P. aeruginosa* strains, the same results obtained by (51,52) blaTEM gene 100% and *tet*A (A) 75.6%, respectively from tested *P. aeruginosa* isolates. In addition, the Aminoglycoside response regulator (arr) gene, a biofilm encoding gene, was detected in all *P. aeruginosa* isolates 100%. A similar result in which *arr* gene was recorded in all *P. aeruginosa* isolates of wild birds (53). Moreover, all the tested *P. aeruginosa* isolates showed to be positive for multidrug-resistant *mex*R gene10/10 (100%). These results agree with the results obtained by El-Deer dash *et al.* (54), who detected *mex*R gene 95% from *P. aeruginosa* isolates.

The fabrication of nanoemulsions with smaller droplet sizes is due to the presence of double bonds in the nonpolar chain of non-ionic surfactants were consistent with the conductivity of the nanoemulsions was increased as the essential oil concentration increased. That result demonstrated that water was during the continuous phase due to the solution conductivity being directly proportional to the number of ions, increasing as the ions increase (55).

Previous studies had found that a high concentration of sulfur compounds of garlic is an essential oil (GC analysis) such as diallyl trisulfide and diallyl disulfide possess good antimicrobial activity (56,57). In agreement with our study, the positive zeta potentials were significantly associated with nano-delivery system uptake across the mucosa (58). Furthermore, garlic oil nanoemulsions of mean size 36.3 nm, of average zeta potential was -26.23 mV (with different ratio of surfactant) was reported, and polydispersity index was 0.527 showed a weak antibacterial effect on some Gram-negative bacteria (59). Our study showed that garlic oil nanoemulsion's low MIC value revealed an antibacterial effect (partial bioactivity) against ten *P. aeruginosa* isolates. The active bacterial cells reduce the non-fluorescent resazurin (blue) to the fluorescent resorufin (pink), which can be further reduced to hydroresorufin, giving a direct, quantifiable measure of bacterial metabolic activity, and the MIC determined through recording of the color change was observed (60). Aeromonas spp. isolates were completely inhibited from growing by the use of chitosan nanoparticle alone or the coating of thyme oil with chitosan nanoparticle at a ratio of 1:1 and 1:0.75 g/ml which can be used as a decontaminant for water tanks and drinkers at the level of

poultry farms as well as a disinfectant product and/or antimicrobial agent for the treatment of a drinking water distribution system (61).

The MIC method was used to assess the sensitivity of *Pseudomonas aeruginosa* to the antibiotics and the nanoparticles ($CoFe_2O_4$ and $NiFe_2O_4$) in vitro which had minimum inhibitor concentrations of 32 g/ml and 16 g/ml, respectively. The appearance/disappearance of bands, an increase in the thickness and clarity of the bands, and other effects of nanoparticles on *P. aeruginosa's* genetic material were noted in the results of the Random Amplification of Polymorphic DNA test (62).

To evaluate the antimicrobial effect of garlic oil nanoemulsion, different resistant genes of *P. aeruginosa* isolates, including *mex*R, *arr*, *tet*A (A), and *bla*TEM, were examined by real-time quantitation PCR relatively, as high degree of bacterial resistant genes downregulation. Results of Real-time PCR agreed highly with phenotypic characterization as the highest degree for downregulation was encountered for all isolates. The results have confirmed the susceptibility of the different isolates to garlic oil nanoemulsion and supported the results obtained by the disk diffusion method. However, different degrees of resistant genes downregulation were recorded because it may be related to microbial response differences and variation in resistant gene expression. Our results revealed that gene expression levels of isolates were affected by garlic oil nanoemulsion. The superior RNA expression level in garlic nanoemulsion treated isolated was significantly different from non-treated or negative isolate control. Many studies recommended garlic oil nanoemulsion as an alternative for antibacterial medicines (63). Furthermore, Relative quantitation real-time PCR results were in remarkable concordance with results obtained by MIC and microdilution susceptibility test for *P. aeruginosa* isolates.

Conclusion

This study showed that a high recovery rate of *P. aeruginosa* at different ages of chickens, especially in dead embryos inside eggshell assessed, is regarded as one of the most critical challenges in the poultry industry. A strict antibiotic policy and the implementation of infection control programs will aid in the reduction of MDR *P. aeruginosa* strains since the broadly used antibiotics usually evolve bacterial resistance or cause harmful influence on the birds' vital organs in expansion to the possible buildups that remain in the poultry meat. Garlic oil nanoemulsion is a novel technology; recently been considered the best alternative to antibiotics in poultry farms. So, the possible use and diverse strategies of garlic nanoemulsion oil extraction for control of *Pseudomonas spp.* The bacterial infection should be held before and after using such application on the farm level. Moreover, In-vitro, garlic oil nanoemulsion could significantly reduce the gene expression levels of MDR *P. aeruginosa*.

Acknowledgments

We would like to thank Prof. Dr. Momtaz A Shahein, Animal Health Research Institute, Agricultural Research Center, Giza, Egypt for facilities of the research study.

Conflict of interest

The authors have no conflict of interest to declare.

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نظرة عامة عن تأثير مستحلب زيت الثوم النانومترية كمضاد لبكتريا السيدوموناس المقاومة للأدوية المتعددة المعزولة من دجاج التسمين

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الخلاصة

لقد كان الهدف من هذا العمل هو التركيز على الخصائص المضادة للبكتيريا لمستحلب نانو الثوم على بعض سلالات السودوموناس المقاومة للمضادات الحيوية المتعددة المعزولة من مزارع الدجاج التسمين وكذلك من المفرخات في محافظتي الشرقية والإسماعيلية في مصر. وقد وجدت نسبة عزل السودوموناس ارجينوزا في ٢١,٣٪ من العينات التي تم فحصها، وكانت نسبة الإصابة ١٥/١١ في الكتاكيت الأصغر من عمر ١-١٠ أيام بينما تم عزلها من العمر الأكبر بنسبة ٥٠/٨ وكذلك وجدت بنسبة ٣٥/١١ في عينات الجنين الميت داخل قشرة البيضة. أيضا سجلت بنسبة ١٥/٢ من المفرخات. وقد سجلت مقاومة المضادات الحيوية نطاقا واسعا يتراوح بين ٦٦,٧ -١٠٠٪ ضد السلالات المعزولة من السودوموناس ارجينوزا حيث حقق المضاد الحيوي التتراسيكلين والسلفاميثوكسازول ميثوبريم أعلى معدلات مقاومة بينما سجل المعدل الأقل مقاومة لكل من المضاد الحيوي: البنسيلين والجنتاميسين. بينما كانت المضادات الحيوية: الانروفلوكساسين والسيبروفلوكساسين والكوليستين هي الأكثر حساسية ضد معزولات السودوموناس ارجينوزا متعددة المقاومة للمضادات الحيوية التي تم فحصها. وقد أثبت تفاعل أنزيم البلمرة المتسلسل وجود الحمض النووي الرايبوزي منقوص الأوكسجين (الدنا) لعشر معزولات من السودوموناس ارجينوزا باستخدام جين *16Sr*، وأثبتت تلك المعزولات أنها الأكثر ضراوة حيث سجلت نسبة وجود جين *opr*L ١٠٠% فيها بالإضافة إلى إيجابية تلك المعزولات أيضا بنسبة ١٠٠% لجينات المقاومة للمضادات الحيوية *tet*A(A) و *bla*TEM و *arr* و *mex*R. وقد أظهرت قيم MIC أن مستحلب نانو الثوم كان فعالاً بتركيزات مختلفة ضد السلالات المعزولة من السودوموناس ارجينوزا التي تم فحصها. وقد وجد انه يحتوي على ٢٩,٦١٪ من مركبات الكبريت النشطة مع ٠,٥٢ ميكروغرام/مل من التركيز المميت الوسطي وحجم ٤٠,٩٤ نانومتر في وجود مؤشر التشتت المتعدد ٠,١٦٥ باستخدام قياس تشتت الضوء الديناميكي حيث وجد بنسبة ١٩,٦±٥,١١ ملي فولط. وأخيرا، فان استخدام مستحلب النانو للثوم قد يكون بديلاً ممتازًا للمضادات الحيوية لمنع نمو وانتشارميكروب السودوموناس ارجينوزا وتقليل المشاكل الناتجة وكذلك خفض مستويات التعبير الجيني لذلك الميكروب في مزارع التسمين.

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