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Highlight on Mobile Genetic Elements Associated with Some Bacteria Isolated from Broiler with Regard to Effect of *Moringa Oleifera* Nanoemulsion on Multidrug Resistance

Nesreen M. Gharib[1], Ashraf A. Abd-El Twad[1], Amal S.A. El Oksh[2*]

[1]Bacteriology, Immunology and Mycology Department Faculty Veterinary Medicine, Benha University, Egypt.

[2]Biotechnology Department, Reference Laboratory for Quality Control of Poultry Production (RLQP), Animal Health Research Institute (AHRI), Sharkia Branch, Agricultural Research Center (ARC), Zagazig 44511, Egypt.

***Correspondence**
Corresponding author: Amal S.A. El Oksh
E-mail address: saidamal19@yahoo.com

Abstract

Poultry bacterial pathogen is a major problem in poultry farms, with serious consequences for poultry and human. Two hundred samples of apparently health and freshly died broiler were collected from different commercial farms at Sharkia governorate, Egypt. *Salmonella* spp. detected in (29%) of examined specimens and serologically identified into *S.* Typhimurium, *S.* Kentucky, *S.* Infantis, *S.* Enteritidis and *S.* Agama with percentages 9.5%, 6.5%, 5%, 4.5% and 3.5%, respectively. Meanwhile, *Pseudomonas* spp. detected in (19%), the most prevalent serotype was *P. aeruginosa* O2, O5, O9, and O11. However, the antimicrobial-resistant strains of pathogens continuously emerge, with ineffective of medical treatments, thus, the isolates were examined for detection of multidrug resistant (MDR), doxycycline exhibited the highest antimicrobial activity against *Salmonella* spp. (55.17%); and ceftriaxone and doxycycline against *Pseudomonas* spp. (52.63%). Uniplex PCR examination for *ampC*, *stn*, *tetA*(B), *Integrase* genes on MGEs were detected in all *Salmonella* spp. isolates, and *mexR*, *tetA* (B), *Integrase* genes in all examined MDR *P. aeruginosa* isolates, meanwhile, *exo*U detected in 80% on MGES. A novel antibacterial strategy was achieved to minimize economic burdens and the health associated with antimicrobial resistance which obliterate pathogens without any adverse effects on poultry and human. Therefore, the application of a trial using *M. Oleifera* nanoemulsion in order to control the multidrug resistant genes expression. These findings demonstrated that *M. Oleifera* nanoemulsion was a good choice to its potential as a drug that can be used against *Salmonella* and *P. aeruginosa* in poultry industry.

KEYWORDS
Salmonella, *Pseudomonas aeruginosa*, *Moringa Oleifera* nanoemulsion, Antibiotic resistance, gene expression, PCR, MGEs

INTRODUCTION

In Egypt and all over the world, poultry infections are a major health issue and economic. Salmonellosis, *Pseudomonas* infection, and other illnesses are linked to the most prevalent infections (Wernicki *et al*., 2017). Salmonellosis has been linked to significant mortality rates in humans, animals, and fowl for more than 125 years. Since the avian gut is a complex, polymicrobial habitat, fowl serve as a major reservoir for *Salmonella* spp. This could put selective pressure on *Salmonella* to change its genetic makeup to be adapted with poultry environment (Foley *et al*., 2013). Acute or chronic chicken salmonellosis costs the poultry business a lot of money because it kills so many early chicks and has a crippling effect that makes other illnesses more likely to develop (Crump *et al*., 2015). *Salmonella* spp. belongs to the Enterobacteriaceae family and has a total of 2600 serotypes and 51 serogroups (Gal-Mor, 2018). Salmonellosis in chickens causes pasty diarrhea, lethargy, dehydration, growth retardation, blindness, and lameness in broiler chicks that are one week old. The main gross abnormalities include hepatomegaly with necrotic foci, splenomegaly, pericarditis, panophthalmitis, persistent yolk sac, and arthritis (Swayne, 2020). Any breed of chicken, regardless of age, is susceptible to typhoid infection. Paratyphoid infection seldom causes death in infants under 3 weeks of age, but the survivors become carriers and spread the organisms into the environment, with mortality may reach to 80% (Abd El-Mohsen and Sherry, 2022). *Salmonella* Enteritidis and *S. enterica* serovar Typhimurium and *S.* Enteritidis contaminated poultry products are the most outbreak for human salmonellosis epidemics (Vose *et al*., 2011).

Pseudomonas aeruginosa is an opportunistic infection that causes severe septicemic or respiratory problems in poultry farms (Walker *et al*., 2002). The mortality rate of freshly dead chicks increased significantly at late stages (Elsayed *et al*., 2016). The difficulty always starts when *P. aeruginosa* enters the incubated eggs from the surrounding environment (Shahat *et al*., 2019). Cell-associated or extracellular components like lipopolysaccharide, alkaline protease, elastase, hemolysins, phospholipase "C" rhamnolipids, biofilm, Pilli, and flagella that increase the pathogenicity and toxicity of the organism are among the numerous factors that contribute to *Pseudomonas*' multifactorial infection process (Fadhil *et al*., 2016). The mechanism of *Pseudomonas* spp. yolk sac infection is that it destroys yolk proteins to cause infection because it is heavily colonized, causing greater tissue damage than they could inflict on the blood, leading to septicemia and large chicken mortalities (Rasamiravaka *et al*., 2015).

Excessive use of antibiotics may facilitate the emergence and spread of resistant *Salmonella*, which can enter humans

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through food or direct contact with infected animals. These resistant organisms might be extremely important in the spread of antibiotic resistance among human infections (Schroeder *et al*., 2002). Antimicrobial resistance (AMR) is a fast-expanding global public health issue. The prevalence of infections caused by antibiotic-resistant bacteria has increased, and certain diseases are now resistant to several different classes of antibiotics. Antimicrobial resistance (AMR) is thought to be responsible for about 500,000 human deaths annually, according to estimates from the Food and Agriculture Organization of the United Nations (FAO) (Reardon, 2014). Increasing the antimicrobial properties of various medicinal plants is essential for treating infectious pathogenic microorganisms that are resistant to treatment. A powerful source of distinct antibacterial actions is thought to be a variety of plants having antimicrobial properties.

Phenotypic resistance genes encoding is transferred among different bacterial through Mobile Genetic Elements (MGEs), such as integrons and plasmids, which are known to potentiate gene gain and loss and a major force that may profoundly change bacterial fitness. This alteration may aid in the genetic adaptability of bacteria to different antibiotic (Vale *et al*., 2022), and some integron types contain a gene conveying resistance of the integron structure (Mazel, 2006).

The native Indian plant *Moringa Oleifera* (*M. Oleifera*) is widely used as a functional food and medicinal plant due to the essential phytochemicals present in its leaves, pods, and seeds. It has several pharmacological properties and a significant nutritional value (Asghari *et al*., 2015). This plant's many parts may be a great source of protein, vitamins, beta-carotene, and other phenolics in addition to possessing vital minerals. The Moringa plant contains zeatin, quercetin, beta-sitosterol, caffeoylquinic acid, and kaempferol in a potent and noteworthy combination (Suleiman *et al*., 2017).

Cell development and differentiation are based on gene expression and regulation. They also enable the cell to adjust to various environments. Gene functions within cells or in multicellular animals can be significantly impacted by gene transcripts by regulating the timing, environment, and amount of expression (Mata *et al*., 2005).

In the light of the above, the current study was designed to investigate the antibacterial examination of some virulent MDR strains of *Salmonella* and *P. aeruginosa* in broilers. Furthermore, we sought to investigate the distributions of antibiotic resistance and virulence genes located on mobile genetic element of MDR isolates. Finally, we endeavored to discuss the effect of *Moringa Oleifera* nanoparticles treatment on down regulating genetic expression.

MATERIALS AND METHODS

Collection of samples

Two hundred samples of visceral organs (heart, lungs and liver) were collected from different commercial farms which apparently health and freshly died broiler with clinical symptoms as (weakness, loss of appetite and poor growth, drooping wings, eyes closed, Watery diarrhea, synovitis) at Sharkia governorate, Egypt (from February to October 2022). All collected samples were tested at the Reference Laboratory of Veterinary Quality Control of Poultry Production (RLQP) Sharkia branch and stored at 4ºC to 8ºC.

Bacterial isolation and identification

Salmonella spp.

Isolation was applied according to ISO 6579.1:2017-AMD2020, all isolates were serotyped according to Patrick and Francois (2007).

Pseudomonas spp.

Collected samples were done according to Quinn *et al*. (2011). *Pseudomonas* spp. isolates were serotyped according to (Homma, 1980).

Antimicrobial susceptibility

According to CLSI (2021) Clinical and Laboratory Standards Institute, the results were interpreted, using the following antimicrobial discs: [oxytetracycline (T) (30 µg), streptomycin (S) (10 µg), doxycycline (Do) (30 µg), colistin (CT) (10 µg), ceftriaxone (CTR) (30 µg), sulfamethoxazole (SXT) (25 µg), ampicillin (AM) (10 µg), norofloxacin (NOR) (10 µg), kanamycin (K) (30 µg) and gentamicin (GEN) (10 µg)]. Multiple antibiotic resistance (MAR) index was calculated, which is useful for screening the spread of

Table 1. Cycling conditions of primers sequences and amplicon sizes.

Target gene	Primers sequences	Amplified segment (bp)	Thermal profile	Reference
ampC	TTCTATCAAMACTGGCARCC CCYTTTTATGTACCCAYGA	550	5 min denaturation at 94°C followed by 35 cycles: 94°C for 30s, 50°C for 40s, 72°C for 45s, final extension step at: 72°C for 10 min	Srinivasan *et al.* (2005)
stn	TTG TGT CGC TAT CAC TGG CAA CC ATT CGT AAC CCG CTC TCG TCC	617	5 min denaturation at 94°C followed by 35 cycles: 94°C for 30s, 59°C for 40s, 72°C for 45s, final extension step at: 72°C for 10 min	Murugkar *et al*. (2003)
tetA(B)	CCTCAGCTTCTCAACGCGTG GCACCTTGCTCATGACTCTT	633	5 min denaturation at 94°C followed by 35 cycles: 94°C for 30s, 50°C for 40s, 72°C for 45s, final extension step at: 72°C for 10 min	Randall *et al.* (2004)
exoU	CCGTTGTGGTGCCGTTGAAG CCAGATGTTCACCGACTCGC	134	5 min denaturation at 94°C followed by 35 cycles: 94°C for 30s, 55°C for 30s, 72°C for 45s, final extension step at: 72°C for 7 min	Winstanley *et al*. (2005)
mexR	GCGCCATGGCCCATATTCAG GGCATTCGCCAGTAAGCGG	637	5 min denaturation at 94°C followed by 35 cycles: 94°C for 30s, 57°C for 40s, 72°C for 45s, final extension step at: 72°C for 10 min	Sánchez *et al*. (2002)
Integrase	TGCGGGTYAARGATBTKGATTT CARCACATGCGTRTARAT	491	5 min denaturation at 94°C followed by 35 cycles: 94°C for 30s, 55°C for 40s, 72°C for 45s, final extension step at: 72°C for 10 min	White *et al*. (2000)
invA	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284	5 min denaturation at 94°C followed by 35 cycles: 94°C for 30s, 55°C for 30s, 72°C for 30s, final extension step at: 72°C for 7 min	Oliveira *et al*. (2003)
16S rRNA	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	618	5 min denaturation at 94°C followed by 35 cycles: 94°C for 30s, 50°C for 40s, 72°C for 45s, final extension step at: 72°C for 10 min	Spilker *et al*. (2004)

bacterial resistance to more than three antibiotics (Christopher *et al*., 2013).

Detection of virulence and antibiotic resistance genes by conventional PCR

Extraction plasmid DNA of twenty MDR *Salmonella* spp. and *P. aeruginosa* isolates were done with QIAprep Spin Miniprep Kit. Virulence and antibiotic resistance plasmid-associated genes were screened by PCR amplifications using different cycling conditions of specific primers which listed in Table 1. The positive amplification products of PCR were tested by agarose gel electrophoresis (Sambrook *et al*., 1989).

Preparation and estimation of MIC of Moringa Oleifera nanoemulsion

Moringa Oleifera nanoemulsion was prepared according to Mahdi *et al*. (2016). The measurement MIC; minimum inhibitory concentration; of *M. Oleifera* nanoemulsion against ten MDR *Salmonella* spp. and *P. aeruginosa* isolates for each, 96-well plates were used. Each well of the column was filled with 50 µl of peptone water broth before column "1" received 50 ul of the *M. Oleifera* nanoemulsion. Using a multichannel pipette, the *M. Oleifera* nanoemulsion from columns 1 through 10 was transferred and mixed twice by successive dilutions. In each well of the column, 50 µl of *Salmonella* spp. or *P. aeruginosa* (1.5 x 10^5 CFU/mL) was injected. They were then incubated at 37°C / 24 hours 30 µl of 0.015% resazurin were added for re-incubated (2-4 hours).

Resistant genes expression by rt-PCR

RNA extraction

From the harvested culture, (0.5 ml; one volume) of the broth was mixed with (1 ml; double volume) of the RNAprotect bacteria reagent (Qiagen, Germany, GmbH) to prevent RNA from degradation. The mixture was vortexed, incubated for 5 min/room temperature, and centrifuged for 10 min / 8000 rpm. Decanting of the supernatant was done. The pellet was then placed in 200 l of TE buffer containing 1 mg/ml Lysozyme (Biochemica, Applichem). Furthermore, (10 µl) β-mercaptoethanol and (700 µl) RLT buffer were added. Following QIAamp RNeasy Mini kit's Enzymatic Lysis of Bacteria protocol instructions, 500 ml of 100% ethanol was added (Qiagen, Germany, GmbH).

Oligonucleotide Primers

As in Table 2, which were supplied by Metabion (Germany).

SYBR green rt-PCR and analysis

A 25 µl experiment including 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of RevertAid Reverse Transcriptase (200 U/L), 0.5 µl of each primer at a concentration of 20 pmol, 8.25 µl of water, and 3 µl of RNA template was used to test the SYBR green rt-PCR primers. The reaction was carried using a Stratagene MX3005P real-time PCR system, which determines the amplification curves and CT values. The thermal profile was performed according to the following steps: reverse transcription at (50˚C/30 min), primary denaturation at (94˚C/15 min), amplification (40 cycles) secondary denaturation at (94˚C/ 15 sec), extension at (72˚C/ 30 sec), dissociation curve (1 cycle) secondary denaturation at (94˚C/ 1 min) and final denaturation at (94˚C/ 1 min). The variation of gene expression carried out according to Yuan *et al*. (2006) using the following ratio: ($2^{-\Delta\Delta ct}$), Whereas ΔΔCt = ΔCt reference – Δct target; ΔCt target = Ct control – Ct treatment and ΔCt reference = Ct control- Ct treatment.

RESULTS

The prevalence of *Salmonella* spp. in all tested broiler samples (29%), the bacteriological examination revealed that 40/200 (20%) of examined samples were *Salmonella* spp. single bacterial infection, while 18/200 (9%) were positive for mixed infection of examined samples infected by *Salmonella* spp. and *Pseudomonas* spp. (Table 3). All isolates were confirmed by PCR through targeting the conserved genes (*invA*, 16srRNA), respectively.

Furthermore, five *Salmonella* isolates serotypes were revealed, of which (39.7%) were *S.* Typhimurium, followed by *S.* Kentucky, *S.* Infantis, *S.* Enteritidis and *S.* Agama with percentages, 22.4%, 10.3%, 15.5% and 12%, respectively, as shown in Table 3.

Moreover, according to Table 3, (19%) of examined broiler samples were positive for *Pseudomonas* spp., as 20/200 (10%) were single infection by *Pseudomonas* spp., and 18/200 (9%) were mixed infection by *Salmonella* spp. and *Pseudomonas* spp.

Pseudomonas spp. isolates were serotyped as *P. aeruginosa* 18/38 (47.4%), *P. fluorescens* 13/38 (34.2%), *P. putida* 5/38 (13.2%), and *P. fragi* 2/38 (5.3%). The most prevalent *P. aeruginosa* serotypes were: O2, O5, O9, and O11, as shown in Table 3, according to slide agglutination test.

Data in Table 4 declared that 55.17%, 43.10%, 37.93%, 32.76% and 29.31 % of examined *Salmonella* spp. were resistant to doxycycline, norfloxacin, ampicillin, ceftriaxone and oxytetracycline, respectively, while the resistance of *Pseudomonas* spp. isolates were 52.63% to ceftriaxone and doxycycline, 50% to streptomycin and oxytetracycline, 47.36% to ampicillin and 42.10% to kanamycin.

Table 2. Primers sequences, target genes and cycling conditions for SYBR green rt-PCR.

Target gene	Primers sequences	Amplification Annealing ˚C/ sec.	Dissociation curve Annealing ˚C/min	Reference
16S rRNA (Housekeeping gene of *Pseudomonas* spp.)	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	50/40	50/1	Spilker *et al*. (2004)
tetA(B)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	50/30	50/1	Randall *et al.* (2004)
mexR	GCGCCATGGCCCATATTCAG GGCATTCGCCAGTAAGCGG	55/30	55/1	Sánchez *et al*. (2002)
16S rRNA (Housekeeping gene of *Salmonella* spp.)	CAGAAGAAGCACCGGCTAACTC GCGCTTTACGCCCAGTAATT	60/30	60/1	Yang *et al*. (2014)
ampC	TTCTATCAAMACTGGCARCC CCYTTTTATGTACCCAYGA	50/40	50/1	Srinivasan *et al.* (2005)

Additionally, 22/58 (37.93%) of isolated *Salmonella* spp. considered as MDR as shown in Table 5, while (28.94%) of *Pseudomonas* spp. isolates were MDR (Table 6).

In this study, all 20/20 (100%) isolates exhibited mobile genetic elements (MGEs) (plasmid and integron class I). Examination of ten MDR *Salmonella* spp. isolates by PCR for *ampC*, *stn*, *tetA*(B) and *Integrase* genes on MGEs were exhibited in 100% for each (Table 7). However, studying the presence of *mexR*, *tetA*(B) and *Integrase* genes of ten MDR *P. aeruginosa* on MGEs revealed (100%) for each, meanwhile, (80%) of MDR *P. aeruginosa* isolates

Table 3. Prevalence and serotypes of *Salmonella* and *Pseudomonas* spp. recovered from broiler chicken farm.

Type of infection	Tested pathogens	Prevalence (%)	Serotypes	Prevalence (%) (no. of samples positive for that serotype)
Single infection	*Salmonella* spp.	40/200 (20%)	*S.* Typhimurium	14/40 (35%)
			S. Kentucky	9/40 (22.5%)
			S. Infantis	3/40 (7.5%)
			S. Enteritidis	7/40 (17.5%)
			S. Agama	7/40 (17.5%)
	Pseudomonas spp.	20/200 (10%)	*P. aeruginosa* O2, O9	9/20 (45%)
			P. fluorescens,	7/20 (35%)
			P. putida	2/20 (10%)
			P. fragi	2/20 (10%)
Mixed infection	*Salmonella* spp.	18/200 (9%)	*S.* Typhimurium	9/18 (50%)
			S. Kentucky	4/18 (22.2%)
			S. Infantis	3/18 (16.7%)
			S. Enteritidis	2/18 (11.1%)
	Pseudomonas spp.		*P. aeruginosa* O5, O11	9/18 (50%)
			P. fluorescens,	6/18 (33.3%)
			P. putida	3/18 (16.7%)

Table 4. Prevalence of antimicrobial resistance pattern of *Salmonella* and *Pseudomonas* spp. Isolates.

Antimicrobial \ Isolated Bacteria	*Salmonella* spp. (n=58)			*Pseudomonas* spp. (n=38)		
	S.	I.	R.	*S.*	I.	R.
Doxycycline (Do)	4 (6.9%)	22 (37.93%)	32 (55.17%)	8 (21.05%)	10 (26.31%)	20 (52.63%)
Norofloxacin (NOR)	18 (31.03%)	15 (25.86%)	25 (43.10%)	10 (26.31%)	8 (21.05%)	20 (52.63%)
Ampicillin (AM)	20 (34.48%)	16 (27.59%)	22 (37.93%)	10 (26.31%)	9 (23.68%)	19 (50%)
Ceftriaxone (CTR)	24 (41.38%)	15 (25.86%)	19 (32.76%)	12 (31.75%)	7 (18.42%)	19 (50%)
Oxytetracycline (T)	30 (51.72%)	11 (18.97%)	17 (29.31%)	14 (36.84%)	6 (15.78%)	18 (47.36%)
Streptomycin (S)	34 (58.62%)	8 (13.79%)	16 (27.59%)	16 (42.10%)	6 (15.78%)	16 (42.10%)
Kanamycin (K)	34 (58.62%)	9 (15.52%)	15 (25.86%)	18 (47.36%)	5 (13.15%)	15 (39.47%)
Gentamicin (GEN)	36 (62.07%)	8 (13.79%)	14 (24.14%)	18 (47.36%)	6 (15.78%)	14 (36.84%)
Colistin (CT)	38 (65.52%)	8 (13.79%)	12 (20.69%)	18 (47.36%)	10 (26.31%)	10 (26.31%)
Sulfamethoxazole –trimethoprim (SXT)	40 (68.97%)	14 (24.14%)	4 (6.90%)	20 (52.63%)	10 (26.31%)	8 (21.05%)

S.: Sensitivity, I.: Intermediate, R.: Resistant

Table 5. Multiple antibiotic resistance (MAR) index and antimicrobial resistance profile of *Salmonella* spp. isolates (n = 58).

Resistance Pattern	Resistance Profile	No. of Isolates	No. of Antibiotics	MAR
I.	Do, NOR, AM, CTR, T, S, K, GEN, CT, SXT	4	10	1
II.	Do, NOR, AM, CTR, T, S, K, GEN, CT	8	9	0.9
III.	Do, NOR, AM, CTR, T, S, K, GEN	2	8	0.8
IV.	Do, NOR, AM, CTR, T, S, K	1	7	0.7
V.	Do, NOR, AM, CTR, T, S	1	6	0.6
VI.	Do, NOR, AM, CTR, T	1	5	0.5
VII.	Do, NOR, AM, CTR	2	4	0.4
VIII.	Do, NOR, AM	3	3	0.3
IX.	Do, NOR	3	2	0.2
X.	Do	7	1	0.1

MAR index = number of antibiotics to which tested strain show resistance / number of antibiotics used for sensitivity assessment, Do: Doxycycline, NOR: Norofloxacin , AM: Ampicillin, CTR: Ceftriaxone, T: Oxytetracycline, S: Streptomycin, K: Kanamycin, GEN: Gentamicin , CT: Colistin, SXT: Sulfamethoxazole –Trimethoprim.

carried *exo*U virulence gene (Table 8).

All data in Table 9, revealed that MIC of *Moringa Oleifera* nanoemulsion 30 mg/mL have the ability to inhibit all examined *Salmonella* spp. and *P. aeruginosa* (100%), meanwhile 15mg/mL inhibit 60% of examined *Salmonella* spp. only. *Moringa Oleifera* nanoemulsion were stopped the growth of *Salmonella* and *P. aeruginosa* isolates, so it was considered the significant and proved effects.

Genes expressions of *tetA*(B) and *ampC* genes of five selected MDR *Salmonella* spp. were examined. The different degrees of resistant genes expression were downregulation by real-time PCR than the untreated by *M. Oleifera* nanoemulsion that ranged from (0.23 to 0.42) for *tetA*(B) gene and from (0.19 to 0.33) for *ampC* gene. Additionally, resistant genes expression of 5 MDR *P. aeruginosa* treated by *M. Oleifera* nanoemulsion were ranged from (0.31 to 0.44) for *tetA*(B) gene and from (0.16 to 0.22) for *mexR* gene (Fig. 1).

DISCUSSION

Egyptian poultry industry has grown to be a key part of Egyptian agriculture. Growing poultry in backyard farms was once

Table 6. Multiple antibiotic resistance (MAR) index and antimicrobial resistance profile of *Pseudomonas* spp. isolates (n = 38).

Resistance Pattern	Resistance Profile	No. of Isolates	No. of Antibiotics	MAR
I.	CTR, Do, S, T, AM, K, NOR, GEN, CT, SXT	8	10	1
II.	CTR, Do, S, T, AM, K, NOR, GEN, CT	2	9	0.9
III.	CTR, Do, S, T, AM, K, NOR, GEN	4	8	0.8
IV.	CTR, Do, S, T, AM, K, NOR	1	7	0.7
V.	CTR, Do, S, T, AM, K	1	6	0.6
VI.	CTR, Do, S, T, AM	2	5	0.5
VII.	CTR, Do, S, T	1	4	0.4
VIII.	CTR, Do	1	2	0.2

MAR index = number of antibiotics to which tested strain show resistance / number of antibiotics used for sensitivity assessment, Do: Doxycycline, NOR: Norofloxacin , AM: Ampicillin, CTR: Ceftriaxone, T: Oxytetracycline, S: Streptomycin, K: Kanamycin, GEN: Gentamicin , CT: Colistin, SXT: Sulfamethoxazole –Trimethoprim.

Table 7. Prevalence of *ampC*, *stn*, *tetA*(B) and *Integrase* genes on MGEs among examined MDR *Salmonella* spp. isolates.

Isolates no.	Serovares	Virulence gene *stn*	Resistant genes		Class I integrin gene *Integrase*
			ampC	*tetA*(B)	
1	*S.* Typhimurium, SI	+	+	+	+
2	*S.* Typhimurium, SI.	+	+	+	+
3	*S.* Typhimurium, MI	+	+	+	+
4	*S.* Enteritidis, SI.	+	+	+	+
5	*S.* Enteritidis, MI	+	+	+	+
6	*S.* Enteritidis, SI	+	+	+	+
7	*S.* Kentucky, MI	+	+	+	+
8	*S.* Infantis, SI	+	+	+	+
9	*S.* Kentucky, MI	+	+	+	+
10	*S.* Agama, SI	+	+	+	+
Total (%)		10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)

SI.: Single infection, MI: Mixed infection, *stn*: enterotoxin virulence gene, *ampC*: β-lactamase resistant gene, *tetA*(B): tetracycline resistant gene.

Table 8. Prevalence of *exo*U, *mexR*, *tetA*(B) and *Integrase* genes on MGEs among examined MDR *P. aeruginosa* isolates.

Isolates no.	Serotypes	Virulence gene	Resistant genes		Class I integrin gene
		*exo*U	*mexR*	*tetA*(B)	*Integrase*
1	*P. aeruginosa* O2, SI	+	+	+	+
2	*P. aeruginosa* O9, SI	+	+	+	+
3	*P. aeruginosa* O5, MI	+	+	+	+
4	*P. aeruginosa* O11, MI	-	+	+	+
5	*P. aeruginosa* O5, MI	+	+	+	+
6	*P. aeruginosa* O11, MI	+	+	+	+
7	*P. aeruginosa* O2, SI	+	+	+	+
8	*P. aeruginosa* O9, SI	+	+	+	+
9	*P. aeruginosa* O2, SI	-	+	+	+
10	*P. aeruginosa* O11, MI	+	+	+	+
Total (%)		8/10 (80%)	10/10 (100%)	10/10 (100%)	10/10 (100%)

SI.: Single infection, MI: Mixed infection, *exo*U: exotoxin virulence gene, *mexR*: multidrug resistant gene, *tetA*(B): tetracycline resistant gene.

simply a traditional activity that supported the well-being of a certain household (FAO, 2017). Because of its low cost, lack of religious barriers, and high quantity of vital amino acids, broiler meat production and consumption developed fast in Egypt. Because of the scarcity of red meat production in Egypt, broiler meat is regarded as an essential source of protein (Hussein *et al*., 2018).

Salmonellosis in birds poses a serious danger to the poultry industry since, which can result a significant financial loss through death with decreased output. According to Table 3, *Salmonella* spp. was detected (29%) of examined samples, the bacteriological examination revealed that (20%) of examined samples with single bacterial infection, while (9%) were positive for mixed infection of examined samples infected by *Salmonella* spp. and *Pseudomonas* spp. The obtained results nearly similar to 28.6% of broiler samples harbor *Salmonella* (Elkenany *et al*., 2019). Comparatively, lower prevalence rate (16%) of broiler *Salmonella* isolates were detected in Egypt (EL-Sheikh, 2018), in Bangladesh 6.2% (Siddiky *et al*., 2021) and in Egypt 11.36% (Abd El-Mohsen *et al*., 2022). A higher isolation rate of *Salmonella* 63.6% in Guangdong, China (Zhang *et al*., 2018). Management, biosecurity, and prophylactic antibiotics used in diverse situations may explain for the variation in *Salmonella* isolation rate among poultrty. Disease transmission via agricultural employees is possible due to lax biosecurity precautions inside farms (El-Sharkawy *et al*., 2017). The detected serotypes were (39.7%) *S.* Typhimurium, followed by *S.* Kentucky, *S.* Infantis, *S.* Enteritidis and *S.* Agama with percentages, 22.4%, 10.3%, 15.5% and 12%, respectively (Table 3). In Turkey, *S.* Enteritidis (21.9%) and *S.* Typhimurium (9.4%) from chicken were shown to be the most common serotypes (Arkali and Çetinkaya, 2020). Similar findings have been recorded in India by Suresh *et al.* (2006) who discovered *S.* Typhimurium and *S.* Enteritidis from poultry compared by other serovar*S.* This study's serovars demonstrate the variety of *Salmonella* spp. in Egyptian native and commercial chicken flocks.

Pseudomonas spp. is an infection that is linked to the environment and can be quite problematic in chicken farms. Any age of bird can contract the disease, although young birds are especially vulnerable. Birds that are under extreme stress or immune compromised, as well as those that are also infected with other viruses and bacteria, are more vulnerable to *Pseudomonas* infection (Miskiewicz *et al*., 2018). In current study, (19%) of examined broiler samples were positive for *Pseudomonas* spp., as (10%) were single infection by *Pseudomonas* spp., and (9%) were mixed infection by *Salmonella* spp. and *Pseudomonas* spp. Nearly similar isolation rate obtained in marketed chicken meat in Egypt (El-Oksh *et al*., 2022). Comparatively lower isolation rate 8% from diseased bird (Betty *et al*., 2007) and 4.57% from diseased and apparently healthy broiler (Abd El-Tawab *et al*., 2014). Numerous variables, such as the type of the samples being studied, poultry immunity, the level of contamination, the nature of the strain, and its virulence, may all contribute to variations in isolation percentages (Khan and Cerniglia, 1994).

According to Table 3, the serological typing of *Pseudomonas* spp. isolates was *aeruginosa* 18/38 (47.4%), *P. fluorescens* 13/38 (34.2%), *P. putida* 5/38 (13.2%), and *P. fragi* 2/38 (5.3%), also *P. aeruginosa* serotypes were: O2, O5, O9, and O11, similar results are reported by Elsayed *et al.* (2016) who detected *P. aeruginosa* (52%) of poultry isolates, El-Oksh *et al.* (2022) who detected *P. aeruginosa* O11, O6 from boiler isolates, meanwhile, a lower prevalence rate of *P. aeruginosa* (8%) was recorded by Betty *et al.* (2007) and Russell *et al.* (1995) who detected *P. aeruginosa* in spoilage of poultry carcasses.

The presence of MDR *Salmonella* considered as a great problem for public health concern. Additionally, there is proof that commercial chicken and red meat in Egypt contain antibiotic residues, which serve as a subnormal dose and hasten antibiotic resistance (Morshdy *et al*., 2013). In current study the isolated *Salmonella* spp. had the ability to resist doxycycline, norofloxacin and penicillin within percentages 55.17, 43.10 and 37.93% (Table 4). Furthermore, 22/58 (37.93%) of isolated *Salmonella* spp. considered as MDR (Table 5). Comparable antibiotic resistant patterns of chicken *Salmonella* isolates in China (Zhang *et al*., 2018), in Egypt (Elkenany *et al*.,2019). Meanwhile higher resistance of *Salmonella* isolates 97.1% and 77.1 to ampicillin and streptomycin in Bangladesh (Alam *et al*., 2020).

The highest resistance rate in examined *Pseudomonas* spp. was 52.63% to ceftriaxone and doxycycline followed by 50% streptomycin and oxytetracycline, (Table 4). Additionally, 28.94% of isolated *Pseudomonas* spp. considered as MDR (Table 6). Previously found variable resistant pattern, in Nigeria the resistance to gentamicin was 55.8%, ampicillin resistance was 81.0%, and ceftriaxone resistance was 79.6% (Ejikeugwu *et al*., 2021). *P. aeruginosa* in Egypt demonstrated 100% and 86.7% resistance to tetracycline and sulfamethoxazole-trimethoprim, respectively (El-Oksh *et al*., 2022). Meanwhile, Higher resistant pattern for *P. aeruginosa* as

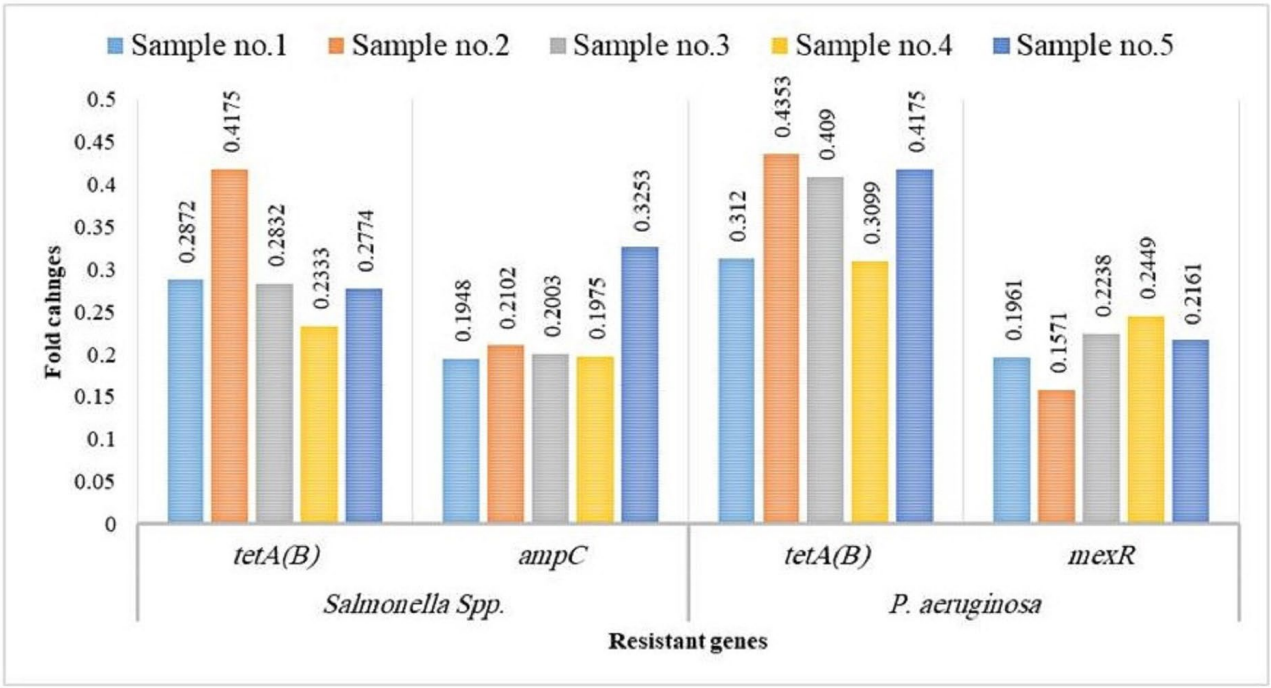

Fig. 1. Effect of *M. oleifera* nanoemulsion on the resistance genes expression on *Salmonella* and *P. aeruginosa* isolates

100% to (ampicillin, tetracycline, and trimethoprim-sulfamethoxazole), 90.7% to streptomycin, 92.6% to ceftriaxone and 18.5% to colistin in Egypt (Algammal *et al*., 2023). The disparity between our findings and other investigations might be related to changes in incubator conditions or to frequent hypermutation among *P. aeruginosa* strains exhibiting varied antibiotic resistance (Maciá *et al*., 2005).

The high frequency of mobile genetic elements (MGEs) seen in industrial-scale poultry operations in developed countries may contribute to heightened antibiotic resistance (AMR) rates in these operations (Nandi *et al*., 2004). Integrons and plasmids have been reported to contain a varied variety of AMR genes, making them a typical route for horizontal spread of AMR and multiple-drug resistance (MDR, and MGEs) may be more essential than bacterial cells in the transmission of AMR genes from poultry to humans (Randall *et al*., 2004).

In this study as shown in Table 7, *ampC* beta-lactamases resistant gene detected in 100% of examined MDR *Salmonella* spp on MGES. The *ampC* detected in 32.5% of examined *Salmonella* isolated from broilers in India (Chowdhury *et al*., 2022). *Salmonella* that produces extended beta-lactamase (ESBL) in chicken varies greatly depending on the exposure to antibiotics, and the exposure further varies with the usage of antibiotics in various geographic locations. Furthermore, although only occasionally observed, plasmids have a substantial role in the horizontal spreading of ESBL genes in poultry system, while the vertical route, which is less important (Nossair *et al*., 2022). The *stn* gene one of the enterotoxin virulent gene in *Salmonella* spp. and detected in (100%) of examined isolates on MGES. Meanwhile, *stn* detected in a previous Egyptian study in 40% of examined *Salmonella* isolated from broiler chickens (Elkenany *et al*., 2019). *tetA* gene code for efflux pumps, which are related to the tet genes in gram-negative bacteria (Schwaiger *et al*., 2010). In current study, *tetA*(B) (tetracycline resistant gene) detected in (100%) of examined *Salmonella* spp on MGES. which similar to the finding of Siddiky *et al*. (2021) who recorded that tetB gene detected in 80-100% of examined poultry MDR *Salmonella enterica* isolates in Bangladesh. The *Integrase* gene detected in 100% of examined *Salmonella* spp. Meanwhile, *Integrase* was found in 20% of poultry isolates (Alam *et al*., 2020). AMR gene dissemination is helped further by mobile genetic elements such as integrons that carry gene cassettes and *Integrase* (White *et al*., 2001). Integrons, in particular class I integrons and AMR genes, were found, as demonstrated by numerous independent publications (Di Cesare *et al*., 2016). Integrons were discovered to be strongly related with resistance antimicrobials such as gentamicin, kanamycin, streptomycin, sulfafurazole trimethoprim, ampicillin, and tetracycline (White *et al*., 2001).

As shown in Table 8, *exo*U (exotoxin virulence gene) detected in 8/10(80%) of examined *P. aeruginosa* isolates on MGEs, it has been shown that *P. aeruginosa exo*U is essential for the release of toxic proteins in the host cells (Habeeb *et al*., 2012). The examined *P. aeruginosa* isolates all tested positive for the *mexR* gene 10/10 (100%) on MGEs, which indicates multidrug resistance. *P. aeruginosa* isolated from poultry in Egypt had a nearly identical detection threshold for *mexR* as 71.4% (Farghaly *et al*., 2017) and 95% (El-Demerdash *et al*., 2000). Furthermore, tetracycline resistance protein, *tetA*(B) and *Integrase* genes detected in 100% of examined *P. aeruginosa* isolates. Lower detection of *Integrase* rate 38% of examined *P. aeruginosa* herbed the gene in Zhenjiang, China (Chen *et al*., 2009).

In current study as in Table 9, low MIC value of *Moringa Oleifera*, nanoemulsion were revealed an antibacterial effect against ten of MDR *Salmonella* and *P. aeruginosa* isolates for each. *M. Oleifera* leaf extract induced 13.14mm inhibition zone around *Salmonella* species isolated from chicken (Allam *et al*., 2016). In addition, 200mg/mL of *M. Oleifera* methanolic extracts considered as MIC for *P. aeruginosa* clinical poultry samples from Nigeria (Bello and Jamiu, 2017). Moreover, several research found that different *M. Oleifera* seed extracts inhibited the growth of *P. aeruginosa* and *Salmonella* Typhimurium (Peter *et al*., 2011). The antimicrobial peptides likely engage in a two-stage interaction with membranes. First off, surfaces with negative charges like phospholipid head groups draw cationic amino acids. Second, the peptide's hydrophobic and positively charged patches engage in interactions with the appropriate aliphatic fatty acids and anionic components. Bacteria are assumed to be killed through leaking of cytoplasmic contents, loss of membrane potential,

Table 9. The minimum inhibitory concentration (mg/mL) of *Moringa Oleifera* nanoemulsion on examined MDR *Salmonella* spp. and *P. aeruginosa* isolates.

Isolates	Control (-ve)	MIC (mg/mL) of *M. Oleifera* nanoemulsion										Control (+ve)
		30	15	7.5	3.75	1.87	0.94	0.47	0.23	0.12	0.06	
S1	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
S2	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
S3	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
S4	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
S5	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
S6	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
S7	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
S8	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
S9	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
S10	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
P1	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
P2	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
P3	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
P4	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
P5	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
P6	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
P7	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
P8	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
P9	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
P10	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

S: *Salmonella* isolates, P: *P. aeruginosa* isolates, -ve: negative, +ve: positive

change in membrane permeability, dispersion of lipids, entrance of the peptide and inhibition of anionic cell components, or activation of autolytic enzymes (Moyo *et al*., 2012).

The resistant genes including *tetA*(B) and *ampC* in *Salmonella* spp. as well as *tetA*(B) and *mexR* in *P. aeruginosa* isolates were examined by Real-time quantitative PCR to assess the comparatively high degree of bacterial resistance gene downregulation which evaluate antimicrobial effect of *M. Oleifera* nanoemulsion. The outcomes have validated the isolates' sensitivity to *M. Oleifera* nanoemulsion. However, varying levels of resistant genes downregulation were observed, possibly reflecting variations in microbial responses and resistant gene expression. Our findings as in Figure 1, showed that *M. Oleifera* nanoemulsion had an impact on the gene expression levels of isolates. When compared to untreated or negative isolate controls, the superior RNA expression level in *M. Oleifera* nanoemulsion treated isolates was considerably higher. Numerous research suggested *M. Oleifera* nanoemulsion as a substitute for antibiotics (Peter *et al*., 2011; Bello and Jamiu, 2017). Relative quantitation real-time PCR findings for *Salmonella* and *P. aeruginosa* isolates were also strikingly consistent with those from MIC and microdilution susceptibility tests.

CONCLUSION

Broiler chicken-isolated *Salmonella* and *P. aeruginosa* are multidrug resistant and carry genes for antibiotic resistance. Because of the potential for transmission of these resistant isolates and their determinants to humans via direct and indirect contact with the birds, the prevalence of multidrug-resistant *Salmonella* and *P. aeruginosa* poses a danger to the public health. The *M. Oleifera* nanoemulsion promise as a medication which used against susceptible bacterial strains.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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