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Antibiogram pattern and molecular characterization of multiple drug resistant Salmonella isolated from different food products in Egypt

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ABSTRACT

Non-typhoidal salmonellosis is a major foodborne infection. The objectives of our study were to investigate the prevalence rates of multiple drug-resistant *(MDR) Salmonella* spp. in various food products, A total of 100 food samples, including 20 samples for each of kariesh cheese, raw milk, chicken fillet, and minced meat, and 10 samples for each of chicken liver and raw meat, were collected from February to July 2022 within different markets in El-Gharbia, Egypt. All samples were subjected to isolation and identification of multiple drug-resistant *salmonella* by cultivation on xylose lysine deoxycholate (XLD) agar, biochemical tests, serological tests, antibiograms, and the Polymerase Chain Reaction (PCR) screen of the virulence gene *(invA*), class 1 integron, and resistant genes (*blaTEM, qnrS,* and *aadA1*). The incidence of *Salmonella* was 2 (2%), and based on molecular serotyping, it was identified as *Salmonella* Kentucky and *Salmonella typhimurium.* All *salmonella* isolates (100%) showed MDR. Variable antibiotic resistance patterns were detected, ranging from 100% for ampicillin, norfloxacin, and gentamicin to 50% for doxycycline and ciprofloxacin. Based on PCR analysis, *invA*, class 1 integron, *blaTEM*, *qnrs*, and *aadA1* were detected in 100% of all identified *Salmonella* isolates.

1. INTRODUCTION

Antimicrobial resistance is one of the most serious worldwide issues today. It leads to millions of mortalities. It also poses a danger to food safety and animal health (WHO, 2021). Animal and poultry farms frequently use antimicrobials as feed additives, meat product preservatives, and methods of preventing and controlling bacterial disease (Harb et al., 2018). Contamination of food items such as raw meat and kariesh cheese with multiple drugs salmonella mainly occurs through fecal contamination during slaughtering and food processing, as salmonella colonizes mainly the gastrointestinal tract (El-Bagoury, Shelaby, and Saied, 2019; Abd-Elghany et al., 2022).

Skimmed milk (Kariesh cheese) is one of the indigenous white soft cheese types in Egypt, with a high nutritive value, easy digestion, and no heat treatment. El-Bagoury, Shelaby, and Saied (2019) have reported raw milk and kareish cheese as ideal media for microbial growth. The same raw meat is rich in water and proteins, which makes it a highly nutritive diet. However, these properties make it a favorable medium for bacterial growth. Interestingly, reports from Egypt indicate a high level of raw meat contamination with multi-drug-resistant salmonella, which could pose a threat to public health (Abd-Elghany et al., 2022).

The misuse and overuse of antimicrobials in various veterinary sectors, particularly poultry farms, has resulted in the emergence and spread of multiple drug-resistant (MDR) Salmonella strains in food (Irani et al., 2018).

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control) classify salmonellosis as the second human zoonotic disease that underlies gastrointestinal illnesses. These illnesses are mainly caused by *Salmonella typhimurium* and *Salmonella entertidis* (EFSA and ECDC, 2019; Sodagari et al., 2019).

Recently, non-typhoidal salmonellosis (NTS) has become a major global foodborne infection caused by the consumption of foods contaminated with *Salmonella* spp. By 12 to 36 hours' post-ingestion of contaminated foods with salmonella (incubation period), symptoms appear as fever, severe diarrhea, nausea, vomiting, and abdominal cramps (Morshdy, 2021). Both antibiotic resistance and virulence factors are required by the organism for survival against host defenses (Ibrahim et al., 2015).

As of late, broad-spectrum ß-lactams and fluoroquinolones are no longer killing *S. typhimurium* and *S. Kentucky*, along with other types of salmonella (Gupta et al., 2019).

Bacterial resistance mechanisms against antibiotics involve enzyme inactivation, decreased cell permeability, and alteration or replacement of the target (Frye and Jackson, 2013). Extended-spectrum beta-lactamase (ESBL) *salmonella* are from relevant resistance groups present in hospitals, farm animals, poultry meat, and meat products. ESBL, with *the blaTEM* and *blaSHV genes* being the most frequently significant encoding genes (Jin and Ling, 2006).

Multiple drug-resistant (MDR) salmonella strains are characterized by the presence of antibiotic-resistant genes that are located on chromosomes and mobile genetic elements such as transposons, integrons, and plasmids (Almeida et al., 2018). Integrons are the main genetic vehicles responsible for disseminating MDR salmonella strains, which consist of two major types, including chromosomal integrons and mobile integrons (MIs). Mobile

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integrons are of five classes (class 1 to 5), and class 1 integron is the most commonly detected class in MDR salmonella strains (Hossain et al., 2019). MDR salmonella dissemination occurs through mobile genetic elements, resulting in healthy animals becoming carriers of antibiotic-resistant bacteria, and these MDR strains can be transmitted to humans through the food chain, which causes significant human infections (Harb et al., 2018).

The present study aimed to investigate the prevalence, antimicrobial resistance pattern, and molecular detection of virulence genes (*invA*), class 1 integrons, and antimicrobial resistance genes (blaTEM, qnrS, and *aadA1*) among identified *Salmonella* serotypes from different products (chicken, meat, milk, and kariesh cheese) in El-Gharbia, Egypt.

2. MATERIAL AND METHODS

2.1 Ethical Approval

The Ethical Committee, Faculty of Veterinary Medicine, Benha University, approved the protocol for the study (Approval No. BVFVTM/13-06-2023).

2.2. Sample collection

Between February and July 2022, a total of 100 samples, including 40 dairy products (20 raw milk and 20 kariesh cheese), 30 chicken products (10 chicken liver and 20 chicken fillet), and 30 meat products (10 raw meats and 20 minced meat), were collected from different markets in Gharbia, Egypt. Each sample was labeled, placed in an icebox, and transferred immediately to the bacteriology laboratory at the Gharbia Directorate of Veterinary Medicine.

2.3. Preparation of samples.

Briefly, samples (25.00 g or mL according to sample status) were placed in stomacher bags containing 225 mL of 1% buffered peptone water (BPW), then homogenized at 3000 rpm for 2 min and incubated at 37.00 ºC for 18 ± 2 h as a pre-enrichment step.

2.4. Isolation and identification of Salmonella spp.

The method of ISO 6579 (International Organization for Standardization, ISO 6579, 2014) was applied for the isolation and identification of *Salmonella* spp. Briefly, 1 mL from each of the pre-enriched sample homogenates was added to 9 mL of Selenite-F-broth (Oxoid, UK), which was used as a selective enrichment medium, and incubated at 41.5 oC for 24 h. A loopful of that enriched culture was surface streaked on the surface of a xylose lysine deoxycholate (XLD) agar (Himedia, India) plate and incubated at 37 C for 24 h. Suspected colonies (red with or without black centers) were purified and sub cultured onto nutrient agar slopes and incubated at 37 °C for 24 h. The purified colonies were subjected to morphological, biochemical, and serological identification.

2.5. Antimicrobial susceptibility testing

The Kirby-Bauer disc diffusion assay was used to evaluate the drug resistance profile against 12 antibiotics (Oxoid®, UK): oxacillin (OXA, 1µg); cefipime (FEP, 30µg); ampicillin (AMP, 10µg); aztreonam (ATM, 10µg); ciprofloxacin (CIP, 5µg); norfloxacin (NOR, 5µg); levofloxacin (LEV, 5µg); gentamycin (CN, 10µg); doxycycline (DO, 30µg); chloromphenicol (C, 30µg); erythromycin (E, 15µg); and clindamycin (DA, 2µg). Assay results were interrupted according to CLSI (Weinstein, 2021). According to Christopher, Hora, and Ali (2013), the Multiple Antibiotic Resistance (MAR) Index calculates the ratio between the number of antibiotics an isolate is resistant to and the total number of antibiotics tested.

2.6. Molecular detection of virulence and antibiotic resistance genes

Plasmid DNA extraction from samples was performed using the QIAprep Spin Miniprep Kits. (Qiagen, Germany, GmbH). The *invA*, Class 1 integron, *blaTEM*, *qnrS*, and *aadA1* genes were amplified as designed, and cycling conditions are shown in Table 1. According to Sambrook et al. (1989). In a 25-µl reaction, the primers were used along with 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer at a concentration of 20 pmol, 5.5 µl of water, and 5 µl of the DNA template. An Applied Biosystems 2720 thermal cycler was used to carry out the reactions. The PCR products were separated by electrophoresis on a 1.5% agarose gel (Applichem, Germany, GmbH). Ethidium bromide (Sigma, USA) was added to the gel and ran for 1.5 hours at 80 volts. Then visualization and photography using a UV transilluminator.

Table (1) Primers sequences, target genes, amplicon sizes and cycling conditions.

Gene	Primer sequence (5'-3')	Length of amplified product	Primary denaturation	Amplification (35 cycles)			Reference
				Secondary denaturation	Annealing (Optics on)	Extension	
invA	F-GTGAAATTATCGCCACGTTCGGGCAA	284bp	94ºC/5min	94ºC/30 sec	50ºC/30sec	72ºC/30sec	Oliveira et al., 2003
	R- TCATCGCACCGTCAAAGGAACC						
Int1	F-CCTCCCGCACGATGATC	280bp			50ºC/30sec	72ºC/45ºC	Kashif et al., 2013
	R- TCCACGCATCGTCAGGC						
blaTEM	F- ATCAGCAATAAACCAGC	516bp			54ºC/40sec	72ºC/45 sec	Colom et al., 2003
	R-CCCCGAAGAACGTTTTC						
qnrS	F- ACGACATTCGTCAACTGCAA	417bp			55ºC/40sec	72ºC/45sec	Robicsek et al., 2006
	R- TAAATTGGCACCCTGTAGGC						
aadA1	F-TATCAGAGGTAGTTGGCGTCAT	484bp			54ºC/40sec	72ºC/45sec	Randall et al. 2004
	R-GTTCCATAGCGTTAAGGTTTCATT						

3. RESULTS

3.1. Prevalence of *Salmonella* in different food products:

After microbiological examination of a total of 100 samples from chicken, milk, and meat, represented by 10 chicken livers, 20 chicken fillets, 20 raw milk, 20 Kariesh cheese, 10 raw meats, and 20 minced meats, by cultivation, isolation, and identification procedures, we identified 2 positive isolates of salmonella species (2%%) from different samples, as shown in Table 2 and Fig 1.

Table (2) Prevalence and distribution of *Salmonella* in different food products.

Sample	Number of Examined sample	No of *S. Typhimirum* isolate	No of S. Kentucky isolate	Total number of isolates	%
Chicken Liver	10	1	1	2	20%
Chicken fillet	20	0	1	0	0
Raw Milk	20	0	0	0	0
Kariesh Cheese	20	0	0	0	0
Raw Meat	10	0	0	0	0
Minced Meat	20	0	0	0	0
Total	100	1	1	2	2%

3.1. *Salmonella* culture and biochemical characteristics

Typical colonies of salmonella were observed on XLD agar as smooth pale pink colonies with a black center, as shown in Fig. 1. salmonella colonies had biochemically urease-negative results, as shown in Fig. 1

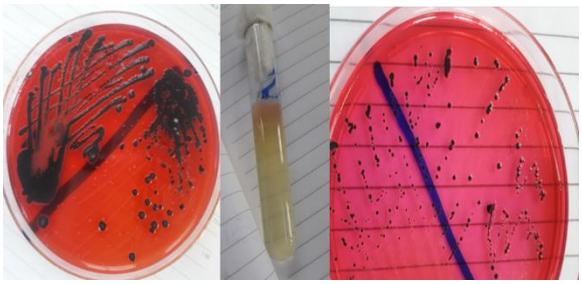

Fig (1) Morphological and biochemical characters of isolated *Salmonella* *(left and right) : Salmonella* morphology on XLD media showing smooth pink colonies with black center . (*Middle*) Urease test showing positive results (remain yellow color)

3.2. Serological findings

Serological analysis revealed two distinct Salmonella strains: (*Salmonella typhimurium*, and *Salmonella Kentucky*)

3.3. Antimicrobial susceptibility findings

Total number of salmonella isolates N=2 tested aganist 12 Antimicrobial discs . All *salmonella* isolates (100%) showed MDR. The recorded results showed high level of resistance about (100%) to norfloxaxin, ampicillin, cefipime, aztreonam, gentamycin, chloramphenicol, erythromycin and clindamycin. Furthermore, moderate resistance to ciprofloxacin, levofloxacin and azithromycin about (50%) . While salmonella isolates showed significant sensitivity to doxycycline about (50%), as shown in Table (3); Fig (2).

Fig (2) Antimicrobial resistance patterns of isolated *Salmonella.*

Table (3) Results of Antimicrobial sensitivity test of *Salmonella*

Antimicrobial Family	Antimicrobial disc	Resistant		Intermediate		Sensitive	
		Number of isolates	%	Number of isolates	%	Number of isolates	%
Quinolones	Ciprofloxacin (CIP)	1	50%	1	50%	0	0
	Norfloxacin (NOR)	2	100	0	0	0	0
	Levofloxacin (LEV)	1	50%	1	50%	0	0
β-lactam	Ampicillin (AMP)	2	100	0	0	0	0
	Cefipime (FEP)	2	100	0	0	0	0
	Aztreonam (ATM)	2	100	0	0	0	0
Aminoglycosides	Gentamycin (CN)	2	100	0	0	0	0
Tetracycline	Doxycycline (DO)	1	50%	0	0	1	50%
Phenicols	Chloromphenicol (C)	2	100	0	0	0	0
Macrolides	Erythromycin (E)	2	100	0	0	0	0
	Azithromycin (AZM)	1	50%	1	50%	0	0
Lincomycin	Clindamycin(DA)	2	100	0	0	0	0

AMP Ampicillin, ATM aztreonam , AZM azithromycin , FEP cefepime, C chloramphenicol, CN gentamicin ,DA clindamycin, DO doxycycline , E erythromycin, CIP ciprofloxacin , NOR norfloxacin ,LEV levofloxacin. .NT-not tested

3.4. Molecular identification findings

PCR findings of *Salmonella* virulence gene *invA*, class 1 integron, and *Salmonella* antibiotic resistance genes Virulence genes *invA and class 1 integron (int1)* were detected in two tested strains (100%), as shown in Fig. 3 (left) and Fig. 3 (right), respectively.

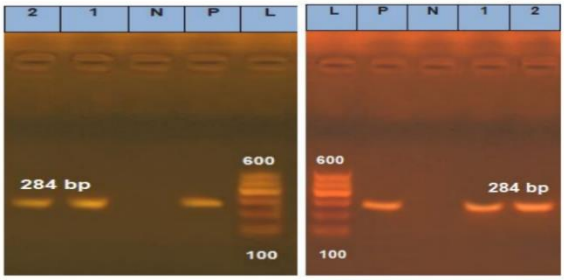

Fig.(3) Ethidium bromide-stained Agarose gel electrophoresis (1.5%) of the amplified *invA* (left) and class 1 integron (right) gene of the isolated *Salmonella*: Lane 2: DNA molecular weight ladder (100bp ladder). P: positive control; N: negative control which is (Nuclease free water). Lanes 1, 2, indicate positive results for *invA* gene (specific band of 284 bp). Lanes 1,2 , indicate positive results for *int1* gene (specific band of 284 bp

The two tested strains (100%), , showed B-lactam resistance genes (blaTEM), fluoroquinolone resistance genes (qnrS), and aminoglycoside resistance genes (aadA1) (fig. 4).

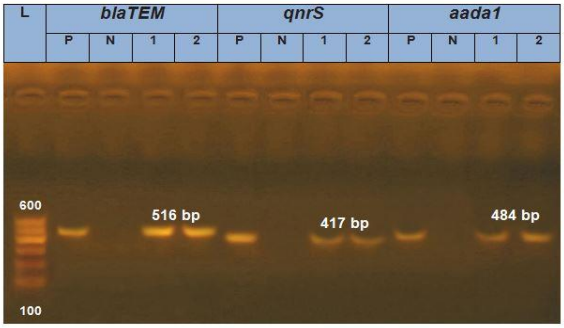

Fig.(4) Ethidium bromide-stained Agarose gel electrophoresis (1.5%) of the amplified *blaTEM* (left),*qnrS* (middle) and *aadA1* (right) gene of the isolated *Salmonella*: Lane 5: DNA molecular weight ladder (100bp ladder). P: positive control; N: negative control which is (Nuclease free water). Lanes 1, 2, indicate positive results for *blaTEM* gene (specific band of 516 bp). Lanes 1,2 indicate positive results for *qnrS* gene (specific band of 417 bp). Lanes 1,2 , indicate positive results for *aadA1* gene (specific band of 484 bp)

4. DISCUSSION

Globally, an increasing concern is developing about the status of antimicrobial-resistant bacterial contaminants in the food chain and their capacity to disseminate and spread to humans. Global public health recognizes MDR salmonella infections as a serious concern (Woh et al., 2021).

In our study, out of 100 examined food products, 2 samples (2%) were contaminated with *Salmonella* spp. Salmonella spp. were mostly isolated from chicken liver (2%) while *Salmonella* spp. weren't detected in the examined chicken fillet, raw milk, kariesh cheese, raw meat, or minced meat (0%). These results agree with Eldin et al. (2023), and Badr et al. (2021), who isolated *salmonella* from chicken by 6.66%, and 7.1% in Egypt., respectively. Our findings are lower than those of Zakaria et al. (2020), who isolated *salmonella* spp. by 23.4% from chicken in Malaysia.

The slide agglutination test identified two isolated salmonella isolates as *S. Kentucky* and *S. typhimurium*. While using PCR screening of *salmonella* isolates for harboring *invA* virulence genes revealed the detection of *invA* in all tested isolates (2/2) (100%), which were similar to Eldin et al. (2023) and Abd-Elghany et al. (2022) but higher than that reported by AlShaheeb et al. (2023), who detected *the invA* gene in *Salmonella spp.* from foodstuffs in Iraq by 69.2%.

Our study revealed contamination of chicken liver with MDR salmonella, which may be due to poor hygienic measures or previously infected chicken. Interestingly, the Food and Agriculture Organization (FAO) forecasts that chicken consumption will reach 41% as a main meat source by 2030. Therefore, the consumption of contaminated chicken meat will represent a high food safety risk. This is primarily due to the ongoing development of multiple antibiotic resistances (Morshdy, 2021).

In line with Alsayeqh et al. (2021), our results show that the widespread and uncontrolled use of antibiotics in poultry farms to treat bacteria or help the birds grow is the main cause of multidrug resistance patterns (MDR) in recovered Salmonella spp. This increases the risk of MDR salmonellae spreading.

Our findings revealed no detection of *salmonella* in Kariesh cheese and raw meat, which agrees with the Centre for Food Safety (2020). According to the Center for Food Safety, in 25 g of Kariesh cheese or ready-to-eat RTE meat, Salmonella shouldn't be detected at all. Nearly the same results were obtained by El-Bagoury et al. (2019), who detected no *salmonella* in Kariesh cheese. In contrast, Abd-Elghany et al. (2022) detected salmonella in raw meat by 25%.

In our phenotypic antimicrobial resistance findings, the two serotypes of *salmonella* were shown to be 100% resistant to ampicillin, cefepime, aztreonam, clindamycin, erythromycin, chloramphenicol, gentamicin, and norfloxacin, and 50% resistant to ciprofloxacin, levofloxacin, azithromycin, and doxycycline. Meanwhile, 50% of isolates were sensitive to doxycycline.

Our antibiogram findings were similar to those of Badr et al. (2021), who showed high resistance to clindamycin and lincomycin 100%, followed by tetracycline, ampicillin, and ciprofloxacin (33%); and Wang et al. (2020), who recorded high resistance to streptomycin (92.7%) and ampicillin (92.7%), followed by tetracycline (40%), and ciprofloxacin (22.33%). In this study, the antimicrobial susceptibility assay results are higher than those of AlShaheeb et al. (2023), who recorded lower resistance for gentamicin by 45.45 and cefotaxime and azithromycin with the same proportion of 36.36%. Our results contradict those of Sodagari et al. (2015), who recorded lower antibiotic resistance to ampicillin (11.7%) and no resistance to 3rd-generation cephalosporin.

In our study, all salmonella isolates from food samples exhibited multidrug resistance to ≥ 3 antibiotics. The multiple antibiotic resistance index (MAR) calculated for *S. typhimurium* and *S.* kentucky was 0. 833. Our MAR results agree with Ali et al. (2019), who recorded 0.537. According to Christopher et al. (2013), isolates with MAR index values higher than 0.2 typically originate from high-risk contaminated farms that typically use antibiotics.

PCR was a significant tool for the accurate detection of MDR *Salmonella*-resistant genes (Eldin et al., 2023). Extended-spectrum B-lactamase enzymes generally mediate B-lactams and third-generation cephalosporin antibiotic resistance mechanisms. (ESBL). Our study also screened the salmonella isolates for encoding (ESBLs) genes, revealing that 100% (2/2) of them have the *blaTEM* gene, agreeing with Badr et al. (2021) and Ahmed (2022), who detected *the blaTEM* gene in 100% (80%) of MDR *salmonella* isolated from chickens in Egypt, respectively. Our results of detecting the flouroquinolone resistance gene (*qnrs*) in identified MDR *salmonella* isolates revealed that (2/2) (100%) of isolates harboring the *qnrs* gene were similar to Badr et al.'s (2021) (100%) detection. The PCR result is acceptable because quinolone resistance is mediated in three ways, including plasmid-mediated resistance genes (*qnr* genes), mutations in the quinolone resistance-determining regions, and efflux pumps mediated by *qepA* genes. (QRDRs) (Hooper and Jacoby, 2015). In this study, the ESBL-producing strain showed resistance to fluoroquinolones, suggesting the possibility that plasmid-encoded fluoroquinolone resistance genes are linked to gene-encoding ESBLs.

Our examined *Salmonella* isolates encode the aada1 gene (aminoglycoside encoding gene) (2/2) (100%), similar to the findings of Ahmed (2022) in Egypt. Interestingly, there is a variation between the phenotypic resistance profile for quinolones and the *qnrS* gene. As some of the antimicrobial resistance genes are silent in bacteria in vitro, these silent genes turn on in vivo and can spread to other bacteria, especially under antimicrobial load (Hooper and Jacoby, 2015). That's why our Norfloxacin and Levofloxacin phenotypic resistance of *Salmonella* isolates was 50% while the *qnrs* gene was encoded in all *Salmonella* isolates (100%), which agrees with El-Sharkawy et al. (2017) recorded phenotypic sensitivity of all *Salmonella* isolates to streptomycin (100%) despite the presence of the streptomycin-resistant gene (*aadA1*) in 50% of the isolates. Integrons are parts of DNA that have a place to attach mobile gene elements (MGE) like transposons and plasmids that carry genes that are resistant to antibiotics. This causes MDR in Salmonella spp. The class 1 integron has been the most widely reported class in the dissemination of resistance genes in MDR Salmonella spp. (Mahdi Askari Badouei et al., 2021).

Our results showed that all *Salmonella* isolates (2/2) (100%) encode *the int1* gene, agreeing with the previous findings of Ahmed (2022) and Mahdi Askari Badouei et al. (2021), who detected class 1 integron in 100% and 95.6% of *Salmonella* isolates, respectively. However, Ali et al., (2019) in Assiut, Egypt, found a lower rate of Class-1 integrons (20%) in Salmonella isolates.

5. CONCLUSIONS

This study revealed the high prevalence of multiple drug resistance (100%) in *Salmonella* spp. isolated from food

products (chicken) in El-Gharbia, Egypt. PCR is a quick and accurate way to find Salmonella genes that are resistant to antibiotics (blaTEM, qnrS, and aadA1) and genes that make the bacteria more likely to cause disease (invA). A lot of the *Salmonella enterica* isolates had both antibiotic-resistant genes and virulence genes (invA). the findings of the present study revealed a strong link between Salmonella MDR patterns and the presence of int-1, which is in charge of spreading genes that make bacteria resistant to antibiotics. Coupled with observing food safety practices and regulating antimicrobial usage are the keys to reducing the incidence of MDR foodborne diseases.

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