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Investigating antimicrobial resistance in *Salmonella* strains isolated from food in Syria

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Abstract

Aim: The emergence of multiple drug resistance in *Salmonella typhi* and *Salmonella paratyphi* poses a significant challenge, necessitating the development of effective treatments to combat these bacteria and reduce infection rates. This *in vitro* study aimed to evaluate the minimum inhibitory concentration (MIC) of various antibiotics against *S. typhi* and *S. paratyphi*.

Methods: Overall, 116 samples were collected from diverse markets in Syria. Molecular techniques, including polymerase chain reaction, were utilized to identify the bacterial genus. Antimicrobial susceptibility testing, employing the disk diffusion method and MIC determination, was conducted to assess the effectiveness of various antibiotics.

Results: Among the isolates, 46 were identified, consisting of 23 *S. typhi* and 23 *S. paratyphi* strains. Resistance to nalidixic acid was observed in 9 out of 23 *S. typhi* and 11 out of 23 *S. paratyphi* isolates. Notably, these nalidixic acid-resistant strains exhibited elevated MIC_{50} values for other fluoroquinolones. Furthermore, most of these resistant isolates, specifically 8 out of 9 *S. typhi* and 11 out of 11 *S. paratyphi*, displayed complete resistance to ciprofloxacin ($MIC_{50} \geq 2$ µg/L).

Conclusion: Based on our findings, only gentamicin, third-generation cephalosporins, and some fluoroquinolones demonstrated efficacy effects against *S. typhi* and *S. paratyphi* isolates in this study.

Keywords: *Salmonella typhi*, *Salmonella paratyphi*, Enteric fever, Fluoroquinolones, Drug therapy, Drug resistance

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Introduction

Typhoid fever, a significant public health concern, disproportionately impacts regions with inadequate access to clean water sources and proper sanitation facilities. This infectious disease affects approximately 11-20 million individuals globally, resulting in approximately 128 000 to 161 000 deaths annually (1). In developed countries, most cases of typhoid fever are acquired through travelling to endemic regions or among immigrants from these areas (2). According to World Health Organization reports, 2 billion people worldwide experience diarrhea, with one-third of these cases attributed to contaminated food sources (3).

Approximately 1.5 million cases of salmonellosis globally occur each year due to direct contact with animals such as dogs, ruminants, and horses (4). *Salmonella*, a bacterium commonly associated with animals, is prevalent in their populations and can be transmitted through their products. This pathogen causes intestinal inflammation in poultry, leading to a high mortality rate (5). In addition, *Salmonella* can result in the deaths of newborn calves (6). While not typically part of poultry's natural intestinal flora, *Salmonella* can be acquired from the environment via live insects, rodents, and contaminated feed. Infected adult animals may not exhibit outward symptoms, and the infection can be spread through pasture, barn bedding, and milking equipment (7). Furthermore, *Salmonella* can also be transmitted through veterinary tools used during animal examination or from the infected animal to the veterinarians (8).

Salmonella is a significant bacterium commonly transmitted through contaminated food and is capable of causing typhoid fever (9). Meat, particularly poultry and pig meat, is a major source of *Salmonella* contamination (10).

The *Salmonella* genus is comprised of *S. bongori* and *S. enterica* species, with *S. enterica* further divided into six subspecies. *S. typhi* and *S. paratyphi* belong to the *S. enterica* subsp. enteric subspecies (11,12).

Antibiotics are crucial in the treatment of typhoid fever. Chloramphenicol was first successfully used to treat patients with typhoid fever in 1948 (13). Chloramphenicol became the preferred drug for treating typhoid fever

until the 1970s, when the first outbreak of infections caused by antibiotic-resistant bacteria was reported (14). There has been a rise in resistance among *S. typhi* strains to chloramphenicol, as well as other drugs such as ampicillin and trimethoprim-sulfamethoxazole. The emergence of resistance to multiple antibiotics poses a significant challenge in managing typhoid fever (15,16). Fluoroquinolones have shown efficacy in treating multidrug-resistant (MDR) typhoid fever (17). Unfortunately, the prevalence of nalidixic acid-resistant *Salmonella* strains, which also exhibit resistance to other fluoroquinolones, has been observed in various countries (18). The detection of *Salmonella* isolates with reduced sensitivity to fluoroquinolones and third-generation cephalosporin, such as ceftriaxone is concerning (19,20).

Salmonella-induced diseases can impose significant stress on individuals, leading to extended periods of work disability. While the mortality rate associated with *Salmonella* infections is relatively low, the treatment costs are high, particularly due to the rising resistance of these bacteria to multiple antibiotics. Research into the effects of certain antibiotics on Syrian *Salmonella* isolates *in vitro* could provide insights for potential i*n vivo* applications, aiming to alleviate symptoms and improve treatment outcomes in the future.

The current *in vitro* study aims to assess the minimum inhibitory concentration (MIC) of various antibiotics against *S. typhi* and *S. paratyphi*. This research is crucial for evaluating the therapeutic significance of these antibiotics in treating infections caused by these bacteria. By determining the effectiveness of different antibiotics against these specific *Salmonella* strains, the study can provide valuable information for guiding treatment strategies and improving patient outcomes.

Materials and Methods

Samples Collection

A total of 116 different samples were collected between March 2022 and April 2023, comprising 46 milk samples, 34 chicken meat samples, and 36 egg samples.

Isolation of Bacteria

The bacteria were isolated using lysine iron agar as a selective medium for intestinal bacteria. Subsequently, the *Salmonella* agar medium was utilized as a differential medium for the *Salmonella* genus, following previously described protocols (21).

Polymerase Chain Reaction

Utilizing the *Salmonella* gene database and Primer 5 software, three specific primer pairs were designed based on the nucleotide sequence of genes. The first primer pair was designed to amplify the *invA* gene specific to the *Salmonella* genus. The second primer pair targeted the *prt* gene responsible for encoding the enzyme involved in paratose sugar synthesis, found in both *S. typhi* and *S. paratyphi* serotypes but absent in other *Salmonella* serotypes. To differentiate between *S. typhi* and *S. paratyphi*, a third primer pair was designed to amplify the *tyv* gene specific to *S. typhi,* encoding the enzyme CDP tyvelose-2-epimerase (Table 1).

Selected isolates were prepared for multiplex PCR. The reaction mixture was prepared and worked under conditions provided in Tables 2 and 3.

Antibiotic Sensitivity Tests

Disc Diffusion Method

The disc diffusion method was employed to test antibiotic susceptibility using different antibiotic tablets (Difco) at the indicated concentrations in µg, including nalidixic acid (30), azithromycin (15), ciprofloxacin (5), norfloxacin (10), tobramycin (10), rifampicin (30), nitrofurantoin (300), imipenem (10), and gentamicin (200).

Minimum Inhibitory Concentration Method

A good broth microdilution method was utilized with 96-well plates (TPP, Switzerland) as per the prior protocol (22). The MIC_{50} values were determined following the recommendations of the Clinical and Laboratory Standards Institute (23). The investigated antibiotics included cefprozil (Bristol-Myers Squibb, New York, USA), ceftazidime (Sigma, St. Louis, USA), ciprofloxacin (Bayer, Istanbul, Turkey), ofloxacin (Sigma), levofloxacin (Sigma), and lomefloxacin (Sigma), sparfloxacin (Sigma). The other antibiotics were gentamicin (Medochemie Limited, Limassol, Cyprus), chloramphenicol (Pfizer, New York, USA), and minocycline (Home Sunshine Pharma, Hefei City, China).

Table 1. Specific Primers Used in PCR

Primer	Nucleotide Sequence
P5` tyv	5`-GAG GAA GGG AAA TGA AGC TTT T-3`
P3` tyv	5`-TAG CAA ACT GTC TCC CAC CAT AC-3`
P5` p r t	5`-CTT GCT ATG GAA GAC ATA ACG AAC C-3`
P3` pr t	5`-CGT CTC CAT CAA AAG CTC CAT AGA-3`
P5` inv	5`-GTA TTG TTG ATT AAT GAG ATC CG-3`
P3` inv	5`-ATA TTA CGC ACG GAA ACA CGT T-3`

Note. PCR: Polymerase chain reaction.

Table 2. Materials used in PCR

Samples	Volume
DNA template (colony)	5 µL
Buffer (10X)	2.5 µL
$MgSO_4$ (50 mM)	1 µL
dNTPs (10 mM)	1 µL
DNA polymerase	0.2 µL
Primers 5` (25 pmole/µL)	3 µL
Primers 3` (25 pmole/µL)	3 µL
H_2O	9.3 µL
Total	25 µL

Note. PCR: Polymerase chain reaction; $MgSO_4$: *Magnesium sulfate;* dNTP: Deoxynucleotide.

Results

Isolation of Bacteria

Overall, 68 samples containing *Salmonella* (78.88%) were obtained when cultured on an iron lysine agar medium. The medium maintained its violet color, and the colonies appeared transparent with or without a dark center. Positive isolates were found in milk (27), chicken meat (12), and eggs (29).

In general, 61 positive samples containing *Salmonella* (70.76%) were obtained when cultured on the *Salmonella* agar medium. The medium turned yellow around the isolates, with the isolates appearing transparent with or without a dark center. The isolates were positive in milk (24), chicken meat (10), and eggs (27).

Polymerase Chain Reaction Analysis

Through multiplex PCR, it was determined that 23 isolates were genotyped as *S. typhi*, with 12 in milk, 5 in chicken, and 6 in eggs. However, 23 isolates were genotyped as *S. paratyphi*, with 6 in milk, 5 in chicken, and 12 in eggs. Finally, 15 isolates were genotyped as *Salmonella* spp., with 6 in milk and 9 in eggs.

The PCR analysis showed that the isolates belonged to different *Salmonella* serotypes based on the bands with specific molecular weights on the agarose gel (Figure 1).

Antibiotic Sensitivity Tests

Disc Diffusion Method

Based on the results (Table 4), all isolates were completely resistant to norfloxacin, nitrofurantoin, and imipenem. Tobramycin and rifampicin demonstrated weak effectiveness on 11 (24%) and 14 (30.4%) isolates, respectively. Azithromycin was the most effective antibiotic, representing susceptibility in 42 out of 46 isolates (91.3%). Gentamicin was effective in 38 out of 46 isolates (82.6%). Ciprofloxacin and nalidixic acid showed moderate effectiveness, with both antibiotics indicating susceptibility in 26 out of 46 isolates (56.5%).

Table 3. PCR Conditions

Initial Denaturation	95°C	5 Minutes
Cycles	35	Cycles
Denaturation	94°C	1 minute
Annealing	60°C	1 minute
Extension	72°C	1 minute
Final extension	72°C	10 minutes

Note. PCR: Polymerase chain reaction.

Antibiotics Minimum Inhibitory Concentration Method

Minimum Inhibitory Concentrations Against Salmonella typhi Isolates Susceptible to Nalidixic Acid

The findings (Table 5) demonstrated that chloramphenicol (MIC_{50mean} = 0.2 µg/mL), gentamicin (MIC_{50mean} = 0.38 µg/mL), and Ceftazidime (MIC_{50mean} = 0.45 µg/mL) were the most effective antibiotics against 14 *S. typhi* isolates susceptible to nalidixic acid. All isolates were sensitive to these antibiotics. In contrast, all isolates were completely resistant to cefprozil and minocycline. In addition, most fluoroquinolones showed high effectiveness against these isolates, except for sparfloxacin, which had moderate effectiveness (MIC_{50mean} = 1.38 µg/mL). The MIC_{50mean} was 0.37 µg/mL, 0.49 µg/mL, 0.28 µg/mL, and 0.31 µg/mL for lomefloxacin, levofloxacin, ciprofloxacin, and ofloxacin,

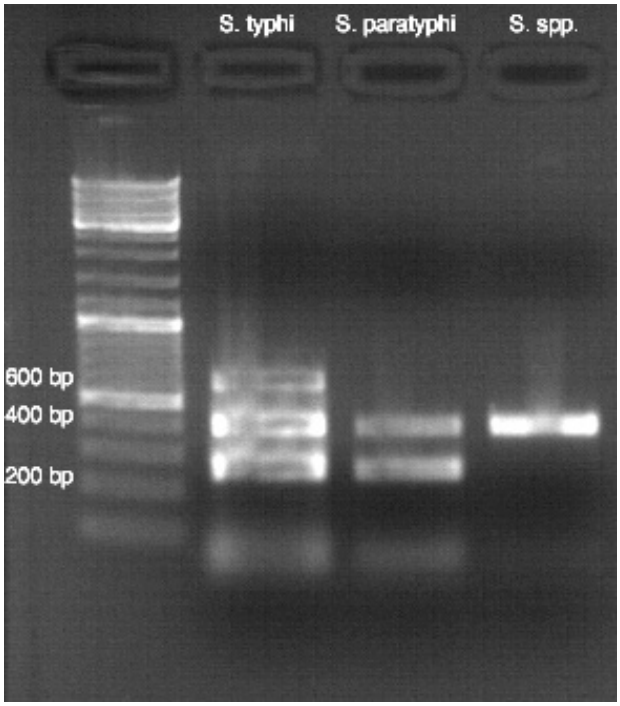

Figure 1. Agarose Gel Electrophoresis Results by Multiplex PCR. *Note*. PCR: Polymerase chain reaction; MW: Standard DNA marker (GeneRuler™ 100 bp). Path 1: Negative control (water), Path 2: *Salmonella typhi* serotype, Path 3: *S. paratyphi* serotype, and Path 4: *Salmonella* genus

Table 4. Number of Susceptible Isolates of *Salmonella typhi* and *Salmonella paratyphi* Seriotypes Using Disc Diffusion Method

Isolate Resource	Isolate Type and Number		Susceptible Isolates (N)								
			Na.ac	Azit.	Cip.	Norf.	Tobr.	Rifa.	Nitr.	Imip.	Gent.
Milk	*Typi*	12	8	10	7	0	3	5	0	0	10
	Paratyphi	6	4	5	4	0	2	3	0	0	5
Chicken meat	*Typi*	5	4	5	4	0	1	1	0	0	4
	Paratyphi	5	4	5	4	0	2	2	0	0	4
Eggs	*Typi*	6	2	6	2	0	1	1	0	0	5
	Paratyphi	12	4	11	5	0	2	2	0	0	10

Note. Na.ac: Nalidixic acid; Azit.: Azithromycin; Cip.: Ciprofloxacin; Norf.: Norfloxacin; Tobr.: Tobramycin; Rifa.: Rifampicin; Nitr.: Nitrofurantoin; Imip.: Imipenem; Gent.: Gentamicin.

respectively.

Minimum Inhibitory Concentrations Against Salmonella typhi Isolates Resistant to Nalidixic Acid

The results (Table 6) revealed that gentamicin (MIC_{50mean} = 0.64 µg/mL) was the most effective antibiotic against 9 *S. typhi* isolates resistant to nalidixic acid. All isolates were sensitive to this antibiotic. All isolates were completely resistant to cefprozil and minocycline, whereas lomefloxacin and levofloxacin showed good efficacy against these isolates (MIC_{50mean} = 1.22 and MIC_{50mean} = 1.28 µg/mL, respectively), and only 6 isolates were susceptible to both antibiotics (67%). On the other hand, 5 isolates were sensitive to chloramphenicol and ceftazidime (56%), 2 isolates were sensitive to sparfloxacin and ofloxacin (22%), and only one isolate was sensitive to ciprofloxacin (11%).

Minimum Inhibitory Concentrations Against S. paratyphi Isolates Susceptible to Nalidixic Acid

Based on the obtained data (Table 7), chloramphenicol (MIC_{50mean} = 0.19 µg/mL), gentamicin (MIC_{50mean} = 0.48 µg/mL), and ceftazidime (MIC_{50mean} = 0.63 µg/mL) were the most effective antibiotics against 12 *S. paratyphi* isolates sensitive to nalidixic acid, and all isolates were sensitive to these antibiotics. On the other hand, all isolates were resistant to cefprozil and minocycline. Moreover, most fluoroquinolones represented high effectiveness against these isolates, and the MIC_{50mean} was 0.51 µg/mL, 0.54 µg/mL, 0.44 µg/mL, and 0.29 µg/mL for lomefloxacin, levofloxacin, ciprofloxacin, and ofloxacin, respectively.

Minimum Inhibitory Concentrations Against Salmonella paratyphi Isolates Resistant to Nalidixic Acid

The results (Table 8) showed that gentamicin (MIC_{50mean} = 0.75 µg/mL) was the most effective antibiotic against 11 *S. paratyphi* isolates resistant to nalidixic acid, and all isolates were sensitive to this antibiotic. Among the quinolones, levofloxacin and lomefloxacin (MIC_{50mean} = 1.14 and MIC_{50mean} = 1.27 µg/mL, respectively) demonstrated moderate efficacy against these isolates. However, 9 (82%) and 8 (73%) isolates were sensitive to levofloxacin and lomefloxacin, respectively. On the other hand, 4 isolates were sensitive to chloramphenicol and sparfloxacin (36%), and three isolates were sensitive to ofloxacin (27%). Finally, all isolates were resistant to cefprozil, ceftazidime, minocycline, and ciprofloxacin.

Table 5. MIC_{50} in *Salmonella typhi* Isolates Susceptible to Nalidixic Acid

Mean of MIC_{50}	Antibiotics	Concentrations of Antibiotics and Number of Isolates Susceptible to Each Antibiotic at Each Concentration							
		0.125	0.25	0.5	1	2	4	8	16
12	Cefprozil	0	0	0	0	0	2	4	8
0.45	Ceftazidime	4	5	1	4	0	0	0	0
0.2	Chloramphenicol	6	8	0	0	0	0	0	0
0.38	Gentamicin	2	8	2	2	0	0	0	0
6.2	Minocycline	0	0	0	1	1	3	5	4
0.37	Lomefloxacin	1	8	4	1	0	0	0	0
0.49	Levofloxacin	1	3	8	2	0	0	0	0
0.28	Ciprofloxacin	5	5	4	0	0	0	0	0
1.38	Sparfloxacin	2	2	3	2	4	1	0	0
0.31	Ofloxacin	5	5	3	1	0	0	0	0

Note. MIC: Minimum inhibitory concentration.

Table 6. MIC_{50} in *Salmonella typhi* Isolates Resistant to Nalidixic Acid

Mean of MIC_{50}	Antibiotics	Concentrations of Antibiotics and Number of Isolates Resistant to Each Antibiotic at Each Concentration							
		0.125	0.25	0.5	1	2	4	8	16
11.56	Cefprozil	0	0	0	0	0	2	2	5
2.78	Ceftazidime	0	0	0	1	4	4	0	0
1.45	Chloramphenicol	0	0	0	5	4	0	0	0
0.64	Gentamicin	0	3	2	4	0	0	0	0
9.33	Minocycline	0	0	0	0	0	3	3	3
1.22	Lomefloxacin	0	0	2	4	3	0	0	0
1.28	Levofloxacin	0	0	5	1	2	1	0	0
3.67	Ciprofloxacin	0	0	0	1	4	2	2	0
2.67	Sparfloxacin	0	0	0	2	5	1	1	0
5.78	Ofloxacin	0	0	0	2	1	2	3	1

Note. MIC: Minimum inhibitory concentration.

Table 7. MIC_{50} in *Salmonella paratyphi* Isolates Susceptible to Nalidixic Acid

Mean of MIC_{50}	Antibiotics	Concentrations of Antibiotics and Number of Isolates Susceptible to Each Antibiotic at Each Concentration							
		0.125	0.25	0.5	1	2	4	8	16
14	Cefprozil	0	0	0	0	0	0	3	9
0.63	Ceftazidime	0	4	3	5	0	0	0	0
0.19	Chloramphenicol	6	6	0	0	0	0	0	0
0.48	Gentamicin	0	3	8	1	0	0	0	0
9.5	Minocycline	0	0	0	0	1	2	5	4
0.51	Lomefloxacin	1	4	4	3	0	0	0	0
0.54	Levofloxacin	0	4	5	3	0	0	0	0
0.44	Ciprofloxacin	2	6	1	3	0	0	0	0
1.07	Sparfloxacin	1	1	3	3	4	0	0	0
0.29	Ofloxacin	4	4	4	0	0	0	0	0

Note. MIC: Minimum inhibitory concentration.

Table 8. MIC_{50} in *Salmonella paratyphi* Isolates Resistant to Nalidixic Acid

Mean of MIC_{50}	Antibiotics	Concentrations of Antibiotics and Number of Isolates Resistant to Each Antibiotic at Each Concentration							
		0.125	0.25	0.5	1	2	4	8	16
15.27	Cefprozil	0	0	0	0	0	0	1	10
8.73	Ceftazidime	0	0	0	0	0	2	7	2
2	Chloramphenicol	0	0	0	4	5	2	0	0
0.75	Gentamicin	0	1	4	6	0	0	0	0
10.18	Minocycline	0	0	0	0	0	4	2	5
1.27	Lomefloxacin	0	0	0	8	3	0	0	0
1.14	Levofloxacin	0	0	5	4	1	1	0	0
6.73	Ciprofloxacin	0	0	0	0	3	3	3	2
2.36	Sparfloxacin	0	0	0	4	5	1	1	0
5.18	Ofloxacin	0	0	0	3	3	2	1	2

Note. MIC: Minimum inhibitory concentration.

Discussion

Typhoid fever continues to pose a real threat to human health in developing countries (17,24-26). Although its severity varies between regions, this disease causes approximately 21.6 million cases of infection and 216.55 deaths worldwide annually (17).

Unacceptably high rates of any infectious disease or a significant increase in the number of infections with this disease strongly motivate greater efforts to prevent it, whether by following health guidelines or performing vaccination campaigns.

The resistance of *S. typhi* to chloramphenicol, amoxicillin, and co-trimoxazole is a challenge to applied therapeutic regimens. Fluoroquinolones have emerged as experimental therapeutic drugs for this disease. Typically, all *S. typhi* isolates are susceptible to ciprofloxacin when using the disk diffusion method approved by the International Council for Laboratory Standards (27). The excessive use of fluoroquinolones in the treatment of typhoid fever has led to an increase in the dose of ciprofloxacin to be used in treatment, demonstrating an increase in the MIC of this drug. Reports show a lack of susceptibility of *S. typhi* isolates to ciprofloxacin in Great Britain, as well as India and its neighboring countries (28,29). Low resistance to ciprofloxacin may result in delayed patient response to treatment or incomplete recovery and serious consequences for successful treatment. Studies suggest the possibility of considering the presence of resistance to nalidixic acid in the disk diffusion method as an indirect indicator of the presence of resistance to quinolones (30). Many studies have shown resistance to several antibiotics used as first-line treatments for *S. typhi* (31,32).

In recent decades, *Salmonella*, especially *S. typhi*, has been able to rapidly develop resistance to antibiotics such as ampicillin, chloramphenicol, and cotrimoxazole, and even to ciprofloxacin (32). MDR enteric fever remains a major problem in all countries (33,34).

In 1993, a significant increase was observed in the number of MDR strains of *Salmonella* in developing countries, in addition to their dramatic resistance to nalidixic acid. In 1998, after 5 years of uncontrolled use of ofloxacin and ciprofloxacin, the proportion of drug-resistant isolates increased, with 87% of strains being resistant to nalidixic acid; this percentage increased to 97% in 2004 (35). The combination of MDR and nalidixic acid resistance is a major problem in such countries, as this reduces treatment options for typhoid fever patients.

The treatment response in patients infected with nalidixic acid-resistant strains is poor, treatment failure rates are high (up to 36%), and the fecal burden of these strains is prolonged for long periods when treated with older generations of quinolones such as ofloxacin (36). Reports from Nepal, India, and Bangladesh of a significant increase in the number of ciprofloxacin-resistant strains sparked the worst drug resistance problem in Asia (37-39). The emergence of isolates of *S. typhi* that represent resistance to ciprofloxacin and third-generation cephalosporin drugs is of great interest to all clinicians in developing countries (39-41).

Although aminoglycosides are not recommended for the treatment of typhoid fever, this group has shown significant activity against *Salmonella* isolates *in vitro*. In addition, a number of cases of gentamicin response have been reported in patients resistant to ciprofloxacin (41).

However, our findings revealed that the most effective drugs against *Salmonella* isolates isolated from meat, chicken eggs, or milk were gentamicin (an aminoglycoside) and chloramphenicol and ceftazidime (third-generation cephalosporins), whether these isolates are sensitive or resistant to nalidixic acid. However, neither cefprozil (a first-generation cephalosporin) nor minocycline demonstrated any significant activity against all isolates. These results are in line with those of Mandal et al (42).

As for fluoroquinolones, our results confirmed that the best drugs in this drug group were levofloxacin (a third-generation fluoroquinolone) and lomefloxacin (a second-generation fluoroquinolone), and to a lesser extent ofloxacin. Conversely, sparfloxacin (the third generation) was the least effective fluoroquinolone. Ciprofloxacin (second generation) showed good activity against isolates sensitive to nalidixic acid only. The results related to this group conform to those that have been published in this regard (43).

Conclusion

MDR was not revealed in this study, *in vitro*. However, varying activities of drugs within the same drug group were observed. Although gentamicin was the most effective drug, it cannot be applied to clinical therapy in any way since aminoglycosides are drugs that specifically affect extracellular bacteria. In addition, despite the differences in fluoroquinolone effects, they generally show good effectiveness. Moreover, third-generation cephalosporins still have significant efficacy against *Salmonella in vitro*.

Authors' Contribution

Conceptualization: Ayman Al-Mariri and Mazen Safi.
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Funding acquisition: Atomic Energy Commission of Syria.
Investigation: Mazen Safi, Samah Qasem, and Laila Al Hallab.
Methodology: Mazen Safi and Ayman Al-Mariri.
Project administration: Mazen Safi, Bassam Al Balaa, and Ayman Al-Mariri.
Resources: Mazen Safi, Bassam Al Balaa, and Ayman Al-Mariri.
Supervision: Mazen Safi and Ayman Al-Mariri.
Validation: Mazen Safi and Ayman Al-Mariri.
Visualization: Mazen Safi, Samah Qasem, and Laila Al Hallab.
Writing-original draft: Mazen Safi and Bassam Al Balaa.
Writing-review and editing: Mazen Safi.

Competing Interests

The authors declare that there are no competing interests.

Ethical Approval

This study was conducted *in vitro*. There is no experiment conducted on humans or live animals.

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