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Salmonella serotypes isolated from frozen chicken meat from Al- Najaf province markets

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Abstract*---Salmonella*** is a zoonotic pathogen that can transfer from animals, contaminated animal products and contaminated water to humans. Non-Typhoidal Salmonella (NTS) are widely spread in wild, domestic animals and human being because it has no host restriction and this presents a broad spectrum of challenges to the public health causing gastroenteritis infections and dissemination of antibiotic resistance. Poultry is one of the most well-known host of NTS without apparent clinical manifestation, but study on overall salmonella population structure is limited. Therefore, a population structure study of salmonella serotypes in chicken meat is important to identify Salmonella serotypes and antibiotic resistance profile in Al-Najaf province. We targeted a novel genomic region that was previously approved for its ability to detect salmonella serotypes, strains of *Salmonella* in frozen chicken flesh, and antibiotic resistance patterns were conducted. 160 specimens from frozen chicken meat during the period extended from October 2020 to December 2020, 30 livers, 49 complete chickens, 25 thighs, and 56 breasts, collected from Al-Najaf province markets 44 (100%). The isolated samples were dominated by *S.* Infantis, 39/44 (88%) and 1/44 (2.2%) *S.*Enteritidis. Two non-salmonella bacteria, *Citrobacter freundii* 2/44 (4.5%) and *Enterobacter cloacae* 2/44 (4.5%) however, sequences from these bacteria were clearly separated from *salmonella* strains. High percentage of antibiotic resistance with tendency of most isolates to show a multi-drug resistance profile to different antibiotic classes was noriced. This study denoted a high prevalence of human pathogenic salmonella strains with multi-drug resistance to different antibiotics present in chicken meat, which shed the light on this food source as a potential of causing public health issues.

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Introduction

Salmonella is one of the most common food-borne diseases in the world, mainly transmitted to human via consumption of animal meat. Chicken meat considered as one of the most often ingested human food resources and detecting the salmonella population structure in this food source is critical for identifying risk factors and monitoring food safety (Ferrari et al., 2019). Salmonellosis is the most common infectious diseases in the world in humans and animals (Chlebicz & Śliżewska, 2018).

Salmonella that have more than 2,600 serovars, each with a different host adaptation and virulence. However, a small percentage of serovars are responsible of causing human infections solely, gastroenteritis infections (Desai *et al.*, 2013). 94 million cases of *NonTyphodial Salmonella* (NTS) gastroenteritis that resulting in causing 155, 000 deaths each year (Jajere *et al.*, 2019).

The broiler sector is an important economic component of the agricultural industry, and poultry meat is an essential component of human diet (Antunes *et al.*, 2016). It is worth mention that *Salmonella* infections with strains that are resistant to several antibiotics are linked to increased morbidity and death, and the global dissemination of such organisms has left doctors with few, if any, therapeutic choices (Hendriksen *et al.*, 2013). Antimicrobial agents are also used in the production of food animals, resulting in the rise of resistant strains of zoonotic pathogens (Rahmani *et al.*, 2013).

A number of molecular targets were employed to identify all bacterial species in an environment for example 16S RNA. However, such method is restricted to detect up to the genus or to some extent to the species level. Meanwhile, a promising DNA region of an Intergenic Sequence Regions ISRs of *rrn*H operon of salmonella genome, showed the ability to detect the salmonella members to the serotype level (Kurylo *et al.*, 2018, Kipper *et al.*, 2019b). So that, this region was chosen in order to identify salmonella population in chicken meat to the level of strains.

2. Material and Methods

2.1. Sample collection

One hundred and sixty of frozen chicken meat samples were collected from local markets during the period extended between October to December 2020. Those samples were as follow: 49 whole chickens, 30 from liver, 25 thighs, and 56 breasts.

The samples were immediately transferred to the microbiology laboratory at Faculty of Veterinary Medicine/ University of Kufa then the samples were

processed immediately by crushing 10 grams of frozen chicken in a sterile pestle and mortar and then adding it to 90 ml of selenite F broth that was incubated at 37°C for 24 hours, following which 1 ml of the combination is removed and added to 9 ml of selenite F broth and incubated at 42°C for 24 hours (Fnes, n.d *et al.*,2019) .

2.2. Identification and biochemical

Bacterial culture were culture on selective media in salmonella and shigella agar and then on Xylose lysine deoxycholate agar. The suspected Salmonella colonies were then subjected to some biochemical testing including the use of Triple Sugar Iron agar and urease test for differentiation from other members of *Enterobacteriaceae* family.

2.3. Targeted amplification of *rrn*H region

The DNA of all isolates was extracted directly from colonies aged 24 hours, as instructed by the DNA extraction kit manufacturing company**.**

The primers used to target *rrn*H region were:

Forword → CGATGCGTTGAGCTAACCG,

Reverse → CAGAAGCGATAACCACGTCG.

The conventional PCR was achieved according to the manufacturing company, the reaction mixture was prepared in a total volume of 25µl. All DNA extracted from *Salmonella* suspected culture were subjected to, covering the end of the gene 23S and at the beginning of the 2,5 didehydrogluconate reductase B gene (Kipper *et al.*, 2019a), as described in table 1.

Table 1. Steps of the PCR thermo cycling.

Steps	°C	M:S	Cycle
Initial Denaturation	94	05:00	1
Denaturation	94	00:30	35
Annealing	60	00:35	
Extension	72	01:00	
Final extension	72	05:00	1
Hold	4		

3. Results and Discussion

3.1. Isolation and Identification of *Salmonella* spp. using conventional.

3.1.1 Cultural characteristics:

Using conventional media, *salmonella* was inoculated on salmonella Shigella agar; colonies of Salmonella appeared colorless with a black center figure (1 a). And then *Salmonella* was planted on Xylose Lysine Deoxycholateand medium, the colonies appeared in a red color with a black center, because *Salmonella* is able to produce hydrogen-sulfide figure (1 b).

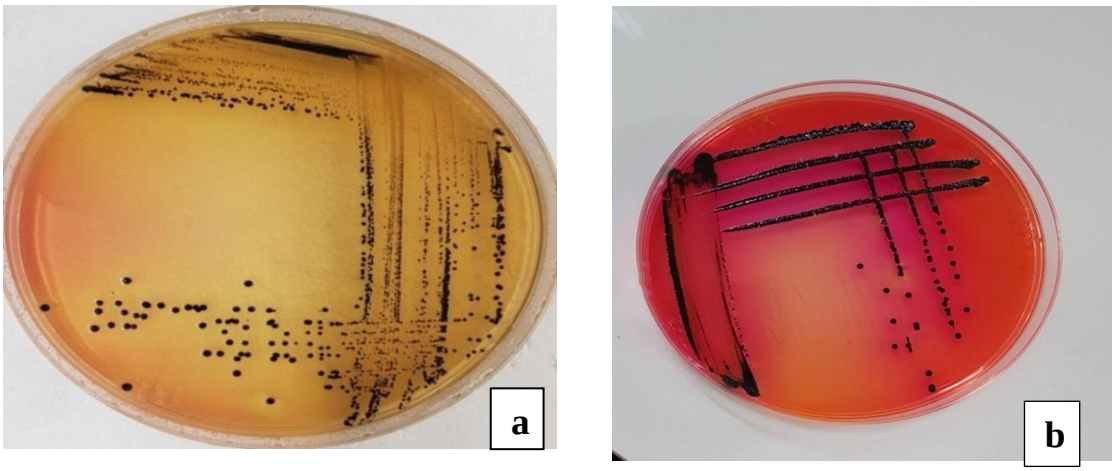

Figure1. Colonial morphology of *Salmonella* isolates on (a) Salmonella Shigella agar, (b) Xylose Lysine Deoxycholate agar.

3.1.2 Biochemical Identification:

The standard positive reaction of *Salmonella* on Triple Sugar Iron, differential medium, was, slant (top) is pink (alkaline) and the bottom is yellow (acidic) with hydrogen sulfide production, as shown in the figure (2a).

The positive reaction of the Urease test was pink, while the negative result showed yellow color. All negative samples on the Urease test were defined as potential *Salmonella spp.* as shown in Figure (2b).

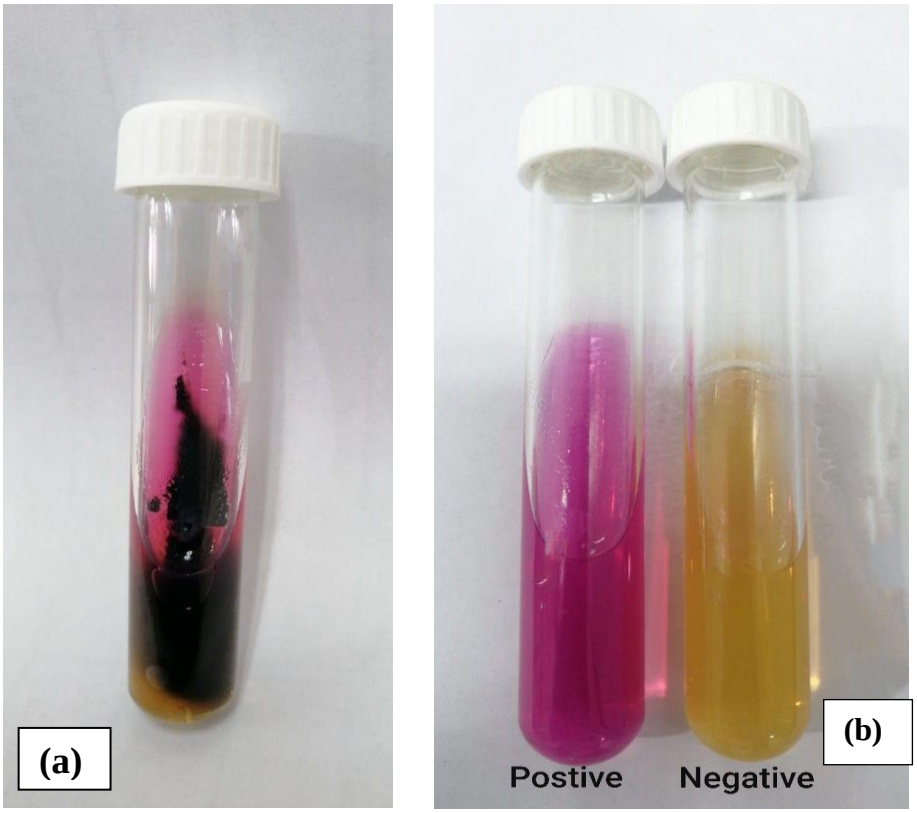

Figure 2. Identification of *Salmonella* on (a) Triple Sugar Iron, (b) Urease test, positive result (pink), negative result (yellow)**.**

3.2. Polymerase chain reaction results of *Salmonella* isolates

Targeted genomic region of all samples was amplified by using specific primers that could produce amplicon size between (600-900) bp, most samples showed a product size of ~ 880 bp. The electrophoresis results are demonstrated in figure 3.

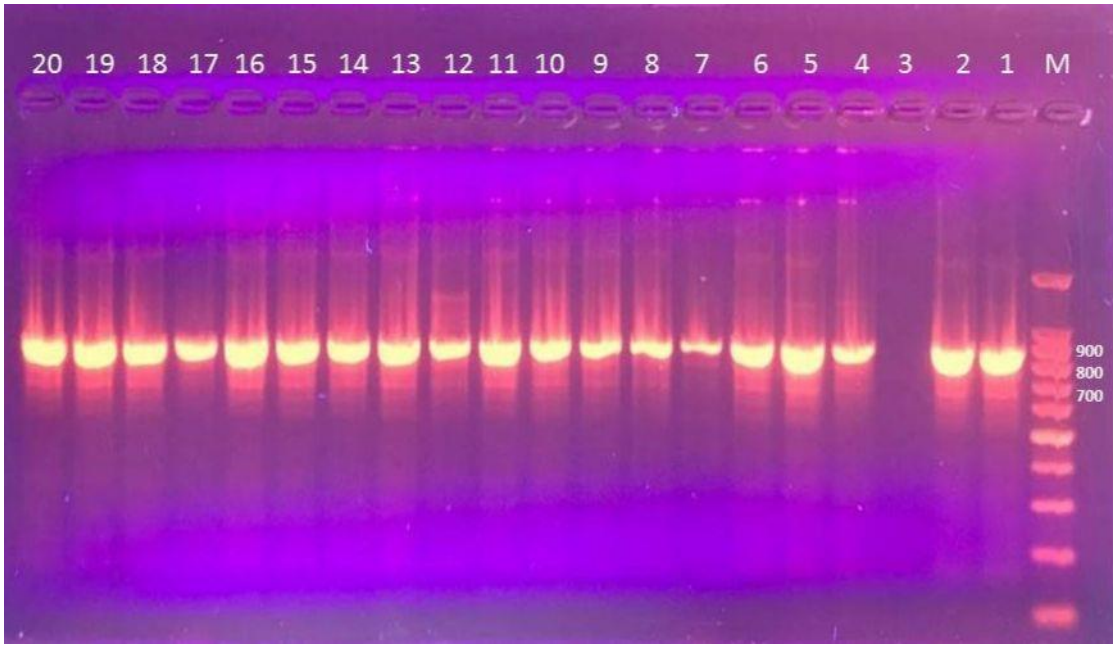

Figure 3. The *rrn*H amplicons on gel electrophoresis among *Salmonella* isolated from frozen chicken meat samples. Lane (M): DNA marker (1500 bp), sample product from (1–20) *rrn*H positive. All samples showed product with molecular weight between ~ 900.

3.3. Sequencing of *Salmonella* isolates

PCR product of the targeted region for all samples. All sequences of the isolates were submitted to the BLASTn search tool of NCBI. Two strains were identified *S.* Infantis 39 (88%) and *S.* Enteritidis 1 (2.2%). However, two non- salmonella bacteria were also had amplified product but with different product size ~ 500 bp, as well as, their sequence were easily detected by BLASTn search against GenBank repositories as *Citrobacter freundii* and *Enterobacter cloacae* in figure (4).

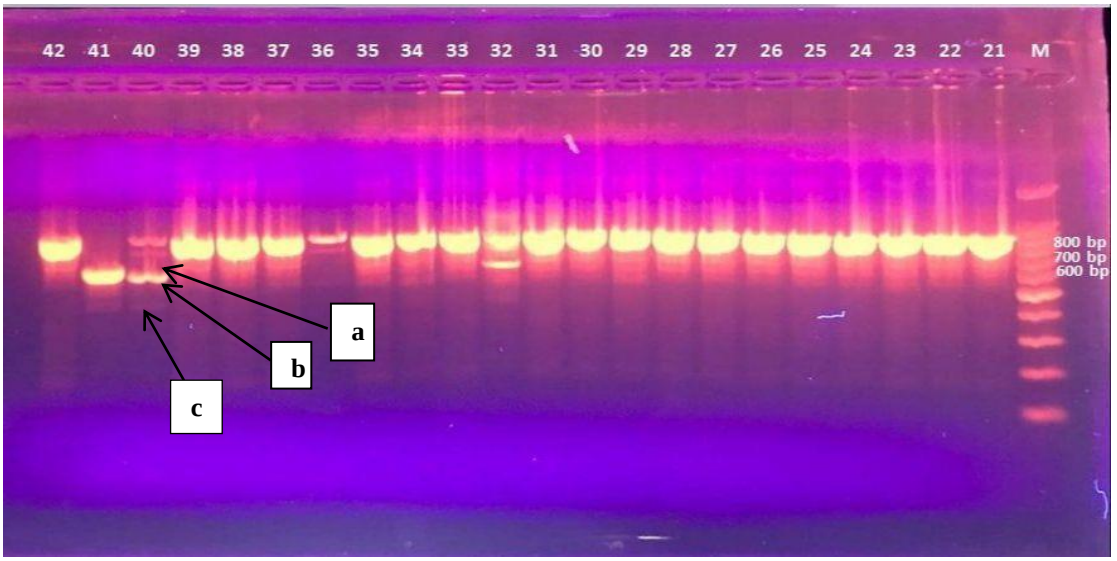

Figure 4. The ISRs amplicons on gel electrophoresis among isolated from frozen chicken meat samples. (a) *S.* Infantis **(b, c)** *Enterobacter cloacae***,** Lane (M): DNA marker (1500 bp), sample product from (21–42) ISRs positive. All samples showed product with molecular weight between 800- 900.

3.4. Characterization of *Salmonella* serotypes

Salmonella strains were discovered among the 42 Salmonella isolates collected from different parts of poultry carcass in table 2. The BLASTn hits to NCBI records were as follows, the predominant serotype was identified as *S.* Infantis strain CVM N17S1509 and only one isolate identified as *S.* Enteritidis strain SE95 as shown in table 3. Such dominant figure of *S.* Infantis from live chicken samples has been also observed by previous work (Abdullah, *et al.*, 2022) These results shed the light on an important new emerging serotype of salmonella that inhabitant poultry in Iraq. The data indicated that *S.* Infantis was the most prevalent serotype, such dissemination might be related to *S.* Infantis capacity to prevent the colonization of other Salmonella serovars, as well as its resistance to antibacterial medications and environmental contaminants (Mejía *et al.*, 2020).

Table 2. Isolation rate of *salmonella* among different parts of the bird.

Parts	Number of Samples	Positive isolates	Isolation rate from the total positive number	**P value**
Breast	56 (35%)	18 (36%)	11.25%	P<0.01
Liver	30 (18.75%)	13 (26%)	8.12%	
Thigh	25 (15.62%)	3 (6%)	1.87%	
Whole chicken	49 (30.62%)	16 (32%)	10%	
Total	160 (100%)	50 (100%)	31.24%	

Table 3: NCBI database search results using BLASTn search tool.

number	Scientific Name	No. of isolates	Query Coverae	Percent Identity
1	***Salmonella* enterica subsp. enterica serovar Infantis strainCVMN17S159**	14	100%	100%
2	***Salmonella* enterica subsp. enterica serovar Infantis strainCVMN17S159**	2	100%	99.8%
3	***Salmonella* enterica subsp. enterica serovar Infantis strainCVMN17S159**	8	100%	99.7%
4	***Salmonella* enterica subsp. enterica serovar Infantis strainCVMN17S159**	4	100%	99.5%
5	***Salmonella* enterica subsp. enterica serovar Infantis strainCVMN17S159**	4	100%	99.4%
6	***Salmonella* enterica subsp. enterica serovar Infantis strainCVMN17S159**	2	100%	98%
7	***Salmonella* enterica subsp. enterica serovar Infantis strainCVMN17S159**	1	98%	99.4%
8	***Salmonella* enterica subsp. enterica serovar Infantis strainCVMN17S159**	1	95%	99.7%
9	***Salmonella* enterica subsp. enterica serovar Infantis strainCVMN17S159**	1	59%	99.3%
10	***Salmonella* enterica subsp. enterica serovar Infantis strainCVMN17S159**	1	55%	99.3%
11	**Salmonella enterica subsp. enterica serovar Enteritidis strain SE95**	1	100%	99.4%
12	**Citrobacter freundii strain E33**	1	100%	99.6%

13	**Citrobacter freundii complex sp. CFNIH9**	1	100%	99.6%
14	**Enterobacter cloacae strain Effluent_2**	1	99%	99.5%
15	**Enterobacter cloacae strain Effluent_2**	1	99%	99.1%

This field work was also unraveled the possibility of amplification of similar genomic regions from other enteric bacteria in chicken, however, it is easily can be distinguish from those of genus *Salmonella* via product size and more accurately by DNA sequence search.

Such homologous genomic region might be represent across genera /spp due to recombination events or horizontal gene transfer (Blake et al., 2021). As a result, relying only on PCR products from this area for proper diagnosis is insufficient; BLASTn searches for sequences from this region are crucial for confirming the serotype of isolated *salmonella*.

4. 5. Phylogenetic analysis

All isolated salmonella sequences clustered in one clade, whereas non-salmonella sequences clustered in a distinct branch, establishing two monophyletic groupings, one for *E. cloacae* and the other for *C. freuidii*, according to nucleic acid sequence clustering (Figure 5). This phylogenetic analysis clearly distinguished salmonella sequences from other sequences. Analysis of phylogenetic trees is a popular technique for classifying species. For the investigation of Salmonella's phylogenetic connections, several genes have been looked at *(Fukushima et al., 2002)*.

The two discovered serotypes in this study were split into two apparent clades, demonstrating the effectiveness of this genomic region in the separation of salmonella serotypes into different clades using ISR sequences of *rrn*H operon. The ability of this approach for serotyping is demonstrated by the apparent division of non-Salmonella sequences into various clades, which is also consistent with the NCBI BLASTn search results.

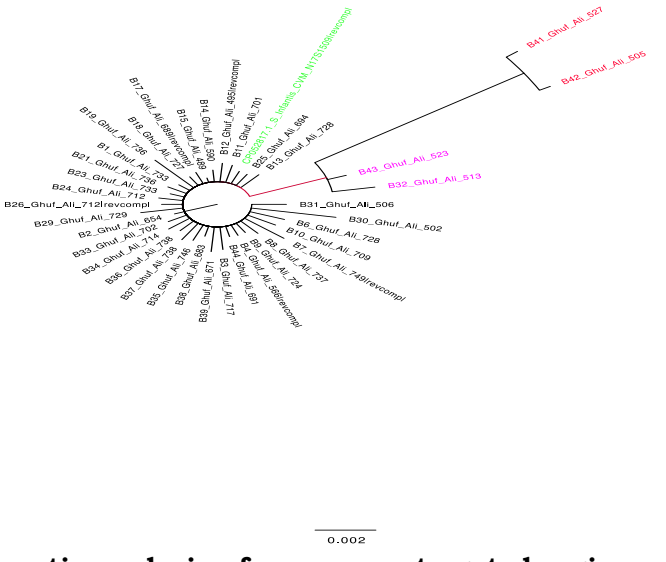

Figure5. Phylogenetic analysis of sequences targeted region amplified from suspected salmonella isolates. Salmonella isolates in this study (black text), reference *S.* Infantis sequence (green text), distant branch (non- salmonella sequences) (red line), *E. cloacae* (red text) and *C. freuidii* (pink text). This tree was constructed using MrBaeys, on aligned sequence by using MUSCLE alignment tool

3. 6. Antibiotic sensitivity profiling

In order to highlight the extent of antibiotic resistant trait of the isolated salmonella serotypes from the frozen chicken meat.

Simple antibiotic diffusion test were achieved and the susceptibility of all 50 isolates to 13 antimicrobial agents were assessed. Remarkably, Most of isolates were highly resistant to different antibiotics belongs to different antibiotic classes: Tetracycline (100%), erythromycin (100%), cephalexin (100%), nalidixic acid (96%), sulfamethoxazole (92%) , amikacin (92%), ampicillin (84%), ciprofloxacin (84%) ,trimethoprim (78%), gentamicin (72%) , amoxicillin(72%) , ceftriaxone (70%) , Chloramphenicol (62%) medicines was determined, and they were classified as resistant, intermediate, or susceptible Figure (6) and Figure (7).

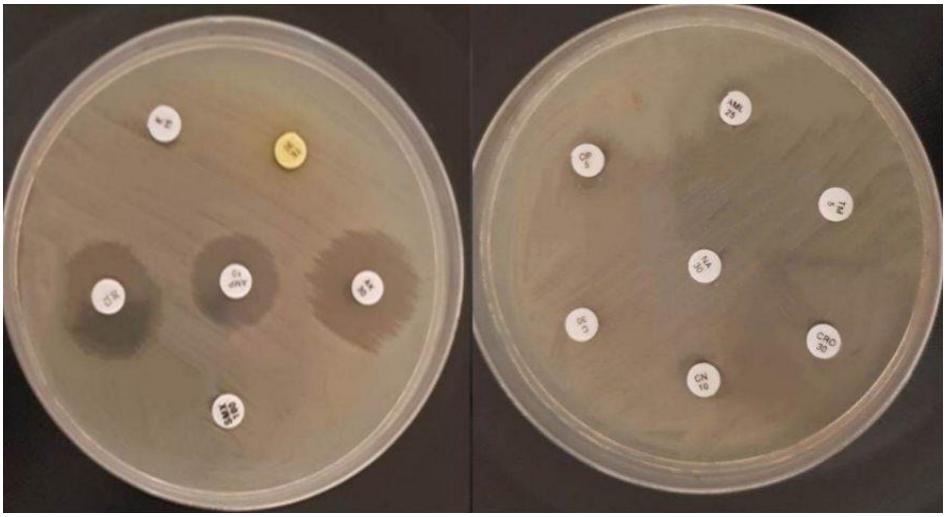

Figure 6. Extreme resistance of some *salmonella* isolates according to the antibiotic susceptibility test.

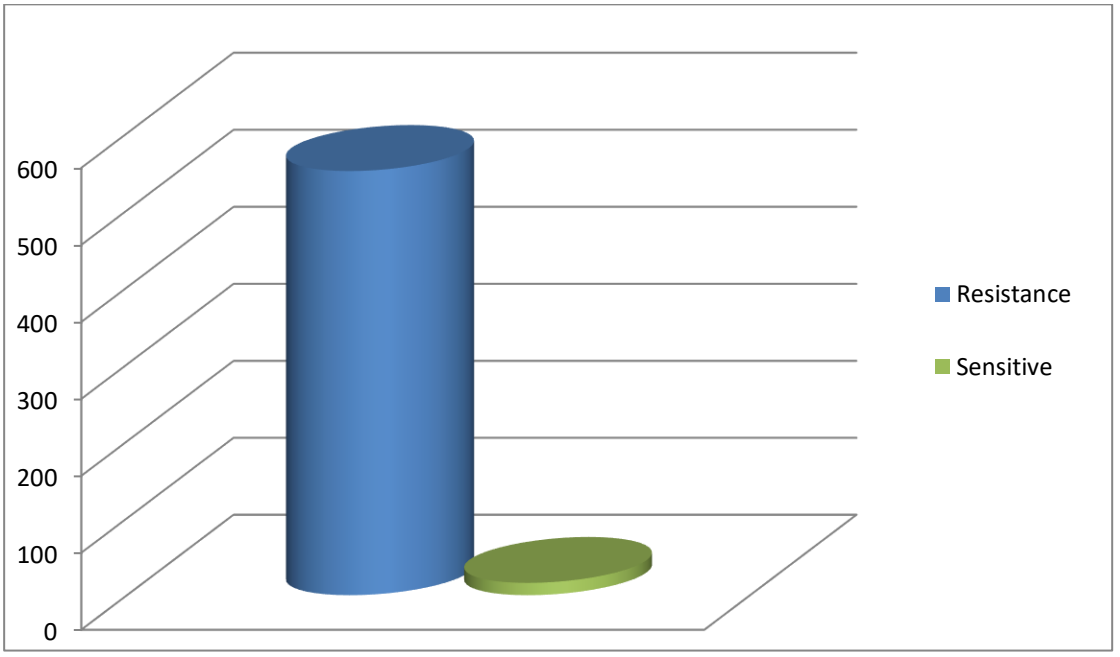

Figure 7. Antimicrobial Resistance patterns of isolated salmonella serotypes. Highly resistantand to the antibiotics tetracycline, ampicillin ,nalidixic acid ,erythromycin, sulfamethoxazole ,ciprofloxacin ,trimethoprim ,Chloramphenicol ,ceftriaxone ,gentamicin ,amikacin ,cephalexin ,and amoxicillin .

The prevalence of susceptibility to each antibiotic tested is presented in Table (4). From the total positive isolates 50 (31.25%) the resistant

pattern as follows: All isolates were resistant to tetracycline, Erythromycin and Cephalexin and very high resistance figure for other antimicrobial agents table (4).

Table 4. Evaluation of antibiotic resistance among *Salmonella* isolated from frozen chicken meat

Antibiotic	**R**	S
Erythromycin	50 (100%)	0
Tetracycline	50 (100%)	0
Cephalexin	50 (100%)	0
Nalidixic acid	48 (96%)	0
Amikacin	46 (92%)	0
Sulfamethoxazole	46 (92%)	2(4%)
Ampicillin	42 (84%)	0
Ciprofloxacin	42 (84%)	0
Trimethoprim	39 (78%)	5(10%)
Amoxicillin	36 (72%)	0
Gentamicin	36 (72%)	3(6%)
Ceftriaxone	35 (70%)	4(8%)
Chloramphenicol	31 (62%)	2(4%)

R- resistant, S- sensitive

All isolates were extensive resistant to antimicrobial drugs as they are resistant to seven or more different classes of antibiotics showing in table 5.

Table 5. Multidrug resistance profile of *Salmonella* serotypes

No. of isolates	No. of antibiotic
13 (26%)	**12**
12 (24%)	**11**
11(22%)	**13**
5 (10%)	**8**
4 (8%)	**10**
3 (6%)	**9**
2 (4%)	**7**

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