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Serotyping, Antibiotic Resistance and Molecular Identification of Salmonella Strains Isolated from Poultry Meat in Rabat, Morocco.

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KEYWORDS

Salmonella, serovars, antibiotic resistance, sequencing, poultry, Morocco.

ABSTRACT

Introduction

Animals serve as a primary reservoir of potential human pathogens, such as Salmonella spp., which is a central concern for biosecurity and food safety.

Methods

The serotyping test was performed after species identification from a pure and fresh culture of Salmonella isolated on Chromagar-Salmonella [14].

Results

The results of the serotyping test showed the presence of the following serovars: *Salmonella Nitra, Salmonella Typhimurium, Salmonella Schwarzengrund, Salmonella Livingstone, Salmonella Enteritidis, Salmonella Bovis Morbificans,*

*Salmonella Aba, Salmonella Cocody, Salmonella Kentucky, and Salmonella Rechevot.*The study of antibiotic resistance in the Salmonella strains isolated in our study showed that 9/18 (50%) of the isolated Salmonella were resistant to Ceftazidime (CAZ 30), 6/18 (30%) to Sulfamethoxazole-Trimethoprim (SXT25), and that all strains were remarkably sensitive to Cefotaxime (CTX30). These tests were followed by sequencing of the 16S rRNA gene of two suspected *Salmonella Typhimurium* strains: S-15 and S-36. This study was designed for the molecular characterization and determination of the phylogenetic relatedness of Salmonella isolates. Compared with the sequence of strain S-15, the sequences of the reference Salmonella strains NR074910.1 is characterized by the highest 16S rRNA similarity values (99.52%). Compared with the sequence of strain S-36, the sequence of the reference Salmonella strain NR074910.1 is characterized by the highest 16S rRNA similarity value (99.2%).

Journal of Chemical Health Risks

www.jchr.org

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Conclusion

In the context of our study, much effort still needs to be made in raising awareness among target audiences (authorities, poultry farmers, vendors, restaurateurs, and consumers) regarding food biosecurity and strengthening epidemiological surveillance, particularly among poultry.

1. Introduction

Animals serve as a primary reservoir of potential human pathogens, such as *Salmonella spp.*[1], which is a central concern for biosecurity and food safety. Detailed characterization of Salmonella is crucial to study the global distribution of Salmonella to prevent future recurrences [2, 3, 4]. Salmonella are bacteria naturally susceptible to several antibiotics. Before 1990, Salmonella strains isolated from human clinical cases showed little or no resistance. The situation took a significant turn with the global epidemic, first in animals, then in humans, caused by Salmonella enterica serotype Typhimurium DT104 penta-resistant to antibiotics (ampicillin, streptomycin/spectinomycin, chloramphenicol/florfenicol, sulfonamides and tetracyclines). Salmonella serotypes are described based on the expression of somatic (O) and flagellar (H) antigens [5]. The presence of numerous Salmonella serovars has been attributed to the ability of Salmonella strains to acquire, recombine, and transfer the fliC (flagellin gene) and rfb (O antigen) cluster genes [6]. Interestingly, Salmonella strains within the same serovar could be polyphyletic and genetically related [7]. Thus, the use of Salmonella serotyping solely for epidemiological tracing has proven inadequate [8]. In recent years, DNA sequencing has been used to determine the evolutionary relationships of different bacteria [9, 10]. 16S rRNA sequencing was used to determine the phylogenetic tree of Salmonella [11]. In congruence, Janda and Abbott [12] showed that genetic analysis of the 16S rRNA gene improves the resolution between the genus and species of Salmonella. It has been sufficiently validated that phylogenetic analyses can be used to determine the relationship between isolated strains and reference pathogenic strains causing serious animal and human diseases [10, 12 ,13].

2. Objectives

The main objective of this study is to characterize Salmonella strains isolated from poultry meat sold in Rabat in order to assess their potential risk to public health.

This is a study that allows for the isolation and identification of Salmonella spp. from poultry meat samples collected in Rabat using conventional bacteriological methods. This study also aims to determine the distribution of Salmonella serotypes isolated through serological and/or molecular serotyping techniques, in order to identify the most prevalent serovars.

3. Methods

Study design

A quantitative study was conducted to determine the serotypes of Salmonella strains detected in poultry samples collected in the Rabat region, as well as their antibiotic resistance to three antibiotics (Ceftazidime (CAZ 30), Sulfamethoxazole-Trimethoprim (SXT 25), Cefotaxime (CTX 30)). This was followed by the molecular identification of two Salmonella strains suspected to be Salmonella Typhimurium.

Sources/measures

Serotyping was performed after species identification from a pure, fresh culture of Salmonella isolated on Chromagar-Salmonella [14].

A total of 18 Salmonella strains and 37 Escherichia coli strains isolated during this study were tested with three different antibiotics to measure resistance. The antibiotics used were: SXT (trimethoprim + sulfamethoxazole) (1.25/23.75 µg), CTX (cefotaxime) (30 µg), CAZ (ceftazidime) (30 µg).

Molecular identification was carried out for 2 suspected Salmonella strains, Salmonella Typhimurium (S-15 and S-36).

Participants

Serotyping was realized at the Medical Bacteriology Laboratory at the National Institute of Hygiene in Rabat.

Journal of Chemical Health Risks

www.jchr.org

JCHR (2026) 16(1), 1754-1765 | ISSN:2251-6727

The measurement of bacterial strain resistance to antibiotics, or antibiogram, was performed in the Medical Microbiology Laboratory at the National Institute of Hygiene.

The DNA samples amplified at the Molecular Biology Laboratory at the Faculty of Sciences in Rabat were sent to the sequencing department of the Functional Genomics Platform of the Technical Support Unit for Scientific Research at the CNRST in Rabat, Morocco.

Experimental-method of serotyping

After verifying that the strain was not self-agglutinating on a drop of saline solution, the following steps were followed:

- Place 1 drop of antiserum on a slide.
- Take 1 loop of the Salmonella culture.
- Suspend these bacteria in the drop of antiserum, taking care to create a homogeneous suspension by gradually adding the bacteria to the serum.
- Shake the slide with a gentle rotating motion.
- Observe the mixture with the naked eye over a dark surface or a concave mirror.

The serotyping test is based on agglutination using Salmonella antiserums (anti-O and anti-H) intended for the serological identification of Salmonella cultures using the slide agglutination method. The sera used for slide agglutination are as follows:

❖ O Antisera

- Polyvalent O Antisera: O Antisera for typing guidance.

These polyvalent O Antisera are not adsorbed. They are intended to guide typing. Their use is particularly recommended for those searching for Salmonella in food products and the environment. The vast majority (around 98%) of Salmonella found in humans and warm-blooded animals possess an O antigen corresponding to the agglutinins contained in OMA and OMB serum [10].

- Monovalent O Antisera: O Antiserum for determining the antigenic formula

These serums are intended for group O identification. They must be used successively in a logical order (polyvalent then monovalent specific to the group), possibly based on specific biochemical characteristics (as in the case of serotypes Typhi and Paratyphi A). Secondary O factors will only be sought in a second step. For example, the following will only be sought:

- Factors O:7 and O:8 if agglutination is observed in serum O:6, 7, 8.

- Factor O:15 if agglutination is observed in serum O:3, 10, 15[5].

❖ H Antisera

- Polyvalent H Antiserum for typing guidance
  Like specific anti-O serum, they are particularly useful when very diverse serotypes need to be identified (food bacteriology, environmental bacteriology).
- Monovalent H Antiserum for determining the antigenic formula

  The same logic will be followed when using these sera as for O sera. For example, the following will be used:

  - Monovalent antisera H:2, H:5, H:6, H:7 if agglutination is observed in the polyvalent H Antiserum 1.
  - Monovalent antisera z10 and z15 if agglutination is observed in the polyvalent H Antiserum E, etc [5].

❖ Antiserum-Vi

The antigen Vi is a heat-labile surface antigen that can mask somatic antigenic activity. It is primarily expressed by S. Typhi strains, and more rarely by S. Paratyphi C strains.

Salmonella possessing this antigen are not agglutinated by antiserum O.

When a bacterium does not agglutinate either the OMA or OMB "mixture," it is recommended to test this bacterium with Vi serum. If the reaction is positive, the bacterial suspension should be heated to 100°C for 30 minutes, then the polyvalent OMA and OMB strains should be retested, followed by the corresponding monovalent strains.

The Kauffman-White-Le Minor table is used to determine the antigenic formula and read the serotyping results [5].

Journal of Chemical Health Risks

www.jchr.org

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Experimental-method of antibiotic resistance

A total of 18 Salmonella strains and 37 Escherichia coli strains isolated during this study were tested with three different antibiotics to measure resistance. The antibiotics used were: SXT (trimethoprim + sulfamethoxazole) (1.25/23.75 µg), CTX (cefotaxime) (30 µg), CAZ (ceftazidime) (30 µg).

The antibiogram was performed according to the following steps:

- Agar inoculation

From a pure bacterial strain culture grown for 18 to 24 hours at 37°C on solid media, collect isolated colonies from agar media using a sterile loop or cotton swab. If possible, collect multiple colonies to avoid selecting an atypical variant. Suspend the colonies in saline and mix to obtain a homogeneous suspension.

Adjust the bacterial density to the 0.5 McFarland standard by adding more saline or more bacteria. A heavy inoculum results in smaller diameters and vice versa.

It is recommended to use a densitometer to adjust the inoculum: in this case, a slight variation can be tolerated, without exceeding the range between 0.4 and 0.6 McFarland.

The bacterial inoculum should ideally be used within 15 minutes of preparation. A sterile cotton swab is dipped into the bacterial suspension and excess liquid is removed by rotating the swab along the sides of the tube. It is important to discard excess liquid to avoid over-inoculation of the agar plates. Swab the entire surface of the agar plate or use a rotary inoculator. The inoculum must be distributed evenly over the entire surface of the agar plate, taking care not to leave any spaces between the streaks**.**

- Depositing the antibiotic-impregnated discs

If the agar plates are left at laboratory temperature for too long before depositing the discs, the bacteria may begin to grow, leading to a false decrease in the size of the zones of inhibition. If multiple plates are to be inoculated with the same inoculum, it is necessary to properly reload the swab between each plate, as previously described. The discs are then firmly placed on the surface of the inoculated, dry agar. Contact with the surface must be tight. Once deposited, the discs cannot be moved, as antibiotic diffusion is very rapid.

The number of discs deposited per Petri dish is limited due to the overlap of the zones of inhibition and to limit interference between antibiotics. It is important that the diameters of the zones of inhibition be measurable. The maximum number of discs depends on the bacteria and the antibiotics, as some produce very large zones for sensitive strains. A maximum of 6 discs is suitable for 90 mm diameter dishes, 12 (or 16) for 150 mm diameter dishes, and 16 for 120 mm square dishes.

The erythromycin and clindamycin discs should be placed 12-20 mm apart from each other to detect inducible lincosamide resistance in staphylococci and streptococci [15].

Experimental-method of molecular identification

Genomic DNA was extracted according to the conventional phenol-chloroform protocol [16]. The amount of DNA was determined using a NanoDrop spectrophotometer (NanoDrop ND2000/2000c, Thermo Fisher Scientific, USA). The 16S rRNA gene was amplified by polymerase chain reaction (PCR), using the two universal primers fD1 and rD1, as previously reported [17]. PCR was performed for 35 cycles (95 °C for 10 s, 56 °C for 40 s, and 72 °C for 2 min), with a final elongation step at 72 °C for 4 min. PCR was performed using a MyTaq mix, according to the manufacturer's specifications (Bioline Reagents Ltd.).

PCR products were analyzed by horizontal electrophoresis on 1% agarose gels (Bioline) stained with ethidium bromide (EtBr) to a final concentration of 0.4% (w/w) (typically 2.5 ml of laboratory stock solution per 100 ml of gel) in 100 ml of trisacetate-EDTA (TAE) buffer at 80 V for 45 min. Profiles were photographed using the ENDURO GDS gel documentation system. Amplified DNA samples were sent to the sequencing department of the Functional Genomics Platform of the Technical Support Unit for Scientific Research at the CNRST in Rabat, Morocco. The sequences obtained were compared to those in GenBank using the BLASTN program [18].

Journal of Chemical Health Risks
www.jchr.org
JCHR (2026) 16(1), 1754-1765 | ISSN:2251-6727

4. Results

Main results of serotyping

The serotyping results of this study showed that 11 serotypes were found in traditional slaughterhouses and supermarkets (Table 1).

Table 1: Salmonella serotypes by sample origin

Serotypes	Sample Origin
Salmonella Nitra	Traditionnel slaughter
Salmonella Schwarzengrund	
Salmonella Enteritidis	
Salmonella Livingstone	
Salmonella Bovis Morbificans	
Salmonella Aba	
Salmonella Cocody	
Salmonella Rechevot	
Salmonella spp.	
Salmonella Typhimurium	Supermarkets
Salmonella Kentucky	

The following table presents the serotypes isolated at the five different sampling sites in our study (Table 2).

Table 2: Salmonella serotypes found at the different sampling sites

Sampling site	Serotype	Number
Océan	*Salmonella Typhimurium*	2
	Salmonella Living stone	1
	Salmonella Aba	1
	Salmonella Enteritidis	1
	Salmonella Nitra	2
Aakari	*Salmonella Living stone*	1
	Salmonella spp.	2
Hassan	*Salmonella Cocody*	1
	Salmonella Bovis morbificans	1
	Salmonella Rechevot	1
	Salmonella Aba	1
	Salmonella Schwarzengrund	1
Agdal	*Salmonella Typhimurium*	1
Les Orangers	*Salmonella Typhimurium*	1
	Salmonella Kentucky	1

Main results of antibiotic Resistance

The study of antibiotic resistance of Salmonella strains isolated in our study showed that 9/18 (50%) of the isolated Salmonella were resistant to Ceftazidime (CAZ 30), 6/18 (30%) to Sulfamethoxazole-Trimethoprim (SXT25), and that all strains were remarkably sensitive to Cefotaxime (CTX 30) (Table 3).

Table 3: Results of antibiotic resistance for Salmonella strains found in traditional slaughterhouses and supermarkets.

Source of Salmonella	Antibiotic	Number of Resistant Strains
Supermarkets	CAZ 30	2
	CTX 30	0
	SXT 25	1
Traditional slaughter	CAZ 30	7
	CTX 30	0
	SXT 25	5

Journal of Chemical Health Risks

www.jchr.org

JCHR (2026) 16(1), 1754-1765 | ISSN:2251-6727

The results of antibiotic resistance for Salmonella strains found at the different sampling sites are shown in the following table (Table 4).

Table 4: Results of antibiotic resistance for Salmonella strains found at the different sampling sites.

Sampling Site	Antibiotic	Number
Ocean	CAZ 30	1
	CTX 30	0
	SXT25	1
Aakari	CAZ30	3
	CTX 30	0
	SXT25	1
Hassan	CAZ30	3
	CTX 30	0
	SXT 25	3
Agdal	CAZ 30	1
	CTX 30	0
	SXT 25	1
Les orangers	CAZ 30	1
	CTX 30	0
	SXT 25	0

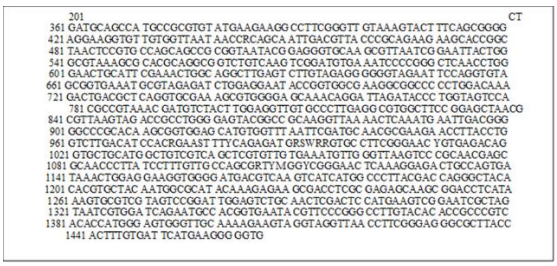
Other results

Antibiotic resistance analysis in this study showed that 8 samples were resistant to ceftazidime (CAZ 30), and 3 samples were resistant to sulfamethoxazole-trimetoprim (SXT 25).

The serotypes antibioresistant to Ceftazidime (CAZ 30) are:

- *S. Typhimurium*
- *S.Bovis Morbificans,*
- *S.Aba.*
- *S.Enteritidis.*
- *S.Nitra.*
- *S.Kentucky.*
- *S.Living stone.*
- *S.Cocody.*

Figure 1: The 16S gene sequence of strain S-15 (total 1107 bp).



The serotypes antibioresistants to Sulfaméthoxazole-triméto**prim**e (SXT 25) are:

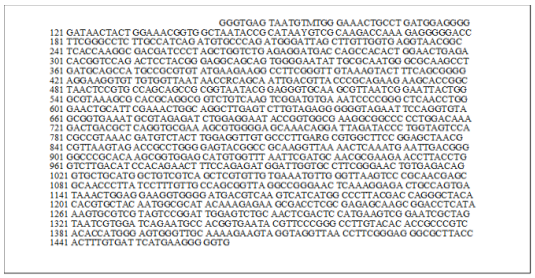
- *S.Schwarzengrund,*
- *S.Typhimurium*
- *S.Aba*

Main results of PCR Product Sequencing

The sequencing results for strains S-15 and S-36 are shown in the following figures (Figure 1, Figure 2).

Sequence similarity study showed the following results (Figure 3, Figure 4)**.**

Figure 2: The 16S gene sequence of strain S-36 (total 1362 bp).



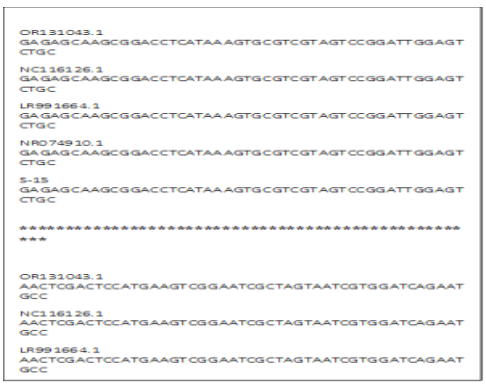


Figure 3: Alignment of the 16S rRNA sequences of the four reference Salmonella typhimurium strains

Journal of Chemical Health Risks

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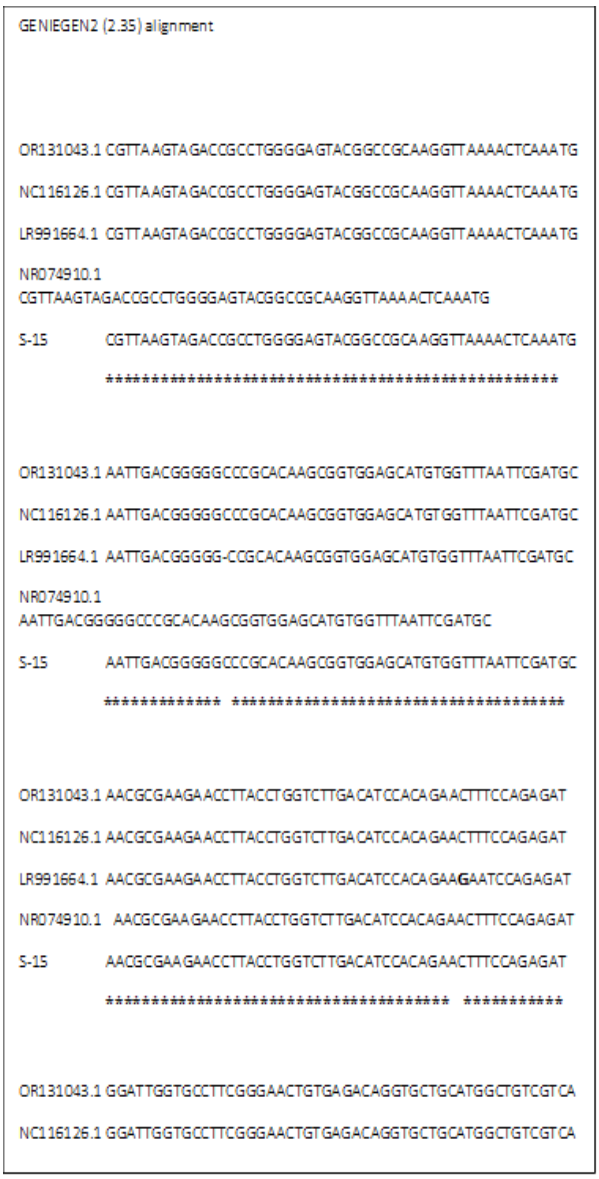
JCHR (2026) 16(1), 1754-1765 | ISSN:2251-6727

NR074910.1, NC116126.1, OR131043.1, LR991664.1, and strain S-15.

Figure 4: Alignment of the 16S rRNA sequences of the four reference Salmonella Typhimurium strains

NR074910.1, NC116126.1, OR131043.1, LR991664.1, and strain S-36.



The similarity study between Salmonella strain S-15 and the 4 reference Salmonella strains with GenBank accession numbers NR074910.1, NC116126.1, OR131043.1, LR991664.1 showed that the highest percentage corresponds to the reference sequence NR074910.1 (Table 5).

Journal of Chemical Health Risks
www.jchr.org
JCHR (2026) 16(1), 1754-1765 | ISSN:2251-6727

Table 5 : Results of the similarity study between the Salmonella S-15 strain and reference Salmonella species based on partial 16S rRNA gene sequencing comparison.

Salmonella isolates and reference species and subspecies	% 16S rRNA gene sequence similarity to Salmonella isolate S-15	Date
Salmonella enterica serovar Typhimurium OR131043.1	99,2	2023
Salmonella enterica serovar Typhimurium NR074910.1	99,52	2022
Salmonella enterica serovar Typhimurium LR991664.1	98,56	2021
Salmonella enterica serovar Typhimurium NR116126.1	99,2	2019

The similarity study between Salmonella S-36 strains and the 4 reference Salmonella strains with GenBank accession numbers: NR074910.1, NC116126.1, OR131043.1, LR991664.1 showed that the highest percentage corresponds to the reference sequence NR074910.1 (Table 6).

Table 6: Results of the similarity study between the Salmonella S-36 strain and reference Salmonella species based on partial 16S rRNA gene sequencing comparison.

Salmonella isolates and reference species and subspecies	% 16S rRNA gene sequence similarity to Salmonella isolate S-36	Date
Salmonella entrrica serovar Typhimurium OR131043.1	98,72	2023
Salmonella entrrica serovar Typhimurium NR074910.1	99,2	2022
Salmonella entrrica serovar Typhimurium LR991664.1	98,25	2021
Salmonella entrrica serovar Typhimurium NR116126.1	98,88	2019

5. Discussion

Serotyping

Compared with the results of our study, a study conducted by Akinyemi et al. in 2018 showed that the percentages of serotypes among 27 Salmonella isolates were 5 (18.5%) S. typhi, 6 (22.2%) S. enteritidis, 9 (33.3%) S. typhimurium, 5 (18.5%) S. cholerasuis, and 1 (3.7%) each of S. arizonae and S. vichow, and were higher than those found in our study, which were 4/18 (22%) S. typhimurium and 1/18 (5.5%) S. enteritidis [19].

Among the 6 serovars identified in a study conducted in Chad in 2017, Salmonella enteritidis (50%) was the most common, followed by S. colindale (33.3%) and S. grampian (16.7%) [20]. In a second study conducted in Chad in 2021, 84 Salmonella isolates belonging to 28 different serotypes were identified. The most frequently encountered serotypes were Salmonella Colindale (19%) and Salmonella Minnesota (18%), followed by S. Havana and S. Riggil (6% each), S. Amager (4.7%), S. Idikan, Mississippi, S. Kottbus and Muenchen (3.6%) [21]. A study showed that the most prevalent serotypes in poultry products are S. Enteritidis in Europe, Asia, Africa and Latin America and S. Typhimurium, S. Sofia and S. Kentucky in North America and Oceania [22]. The 16S rDNA sequences obtained in this work could be used to improve diagnostic tools for members of the genus Salmonella based on molecular genetics.

Antibiotic resistance

Salmonella resistant to third-generation cephalosporins (3GCs) and ciprofloxacins remains a major public health problem. This is especially true given that the treatment of severe human salmonellosis relies primarily on two antibiotics (3GCs and fluoroquinolones), and only one for children (3GCs). It is clear that reducing the use of

Journal of Chemical Health Risks

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JCHR (2026) 16(1), 1754-1765 | ISSN:2251-6727

3GCs in animals is one of the important levers for reducing the prevalence of these enzymes in animal Salmonella. Indeed, European Commission Regulation No. 1831/2003 banned the use of antibiotics as growth promoters in livestock farming in the European Union in order to limit the selection and multiplication of multi-resistant strains [23].

In a 2018 study, 460 patients were selected. 144 (31.30%) positive blood cultures were observed. Salmonella spp was the most isolated germ (41.66%). The Salmonella spp strains isolated in Bukavu are sensitive to ciprofloxacin (91.7%), ceftazidime (81.7%), ceftriaxone (80%), norfloxacin (80%), amikacin (76.6%), and cefuroxime (73.3%). They remain resistant to other antibiotic molecules [24].

The distribution of the 332 strains was: 147 (44.3%) Salmonella Typhi, 97 (29.2%) Salmonella Typhimurium, 74 (22.3%) Salmonella Enteritidis, and 14 (4.2%) Salmonella spp.. The proportions of strains resistant to chloramphenicol, amoxicillin, and cotrimoxazole (first-line antibiotics) increased from 33%, 46%, and 57% in 1998–2002 to 73%, 79%, and 82% in 2003–2004, respectively ($p < 0.0001$). Of the 164 Salmonella strains isolated between 2003–2004, 108 (72%) were resistant to both chloramphenicol, cotrimoxazole, and amoxicillin. The percentage of strains resistant to ciprofloxacin, aminoglycosides, or ceftriaxone was less than 10% [25].

No strain tested showed effective beta-lactamase activity against third-generation cephalosporins. Combined resistance to two critically important antimicrobials—fluoroquinolones and third-generation cephalosporins for Salmonella and fluoroquinolones and macrolides for Campylobacter—remains low. These critically important antimicrobials are frequently used to treat serious Salmonella and Campylobacter infections in humans [26].

Since 2012, observational surveillance of antimicrobial resistance and antibiotic use in the community has been conducted: data on antimicrobial resistance in E. coli isolates were collected from 11 private laboratories, and consumption data were collected from the three major health insurance companies. A significant decrease in the prevalence of resistance to 3GC (from 5.6% to 4.2%; $P < 0.001$), nalidixic acid (from 16.7% to 14.8%; $P = 0.004$), and ciprofloxacin (from 10.9% to 8.1%; $P < 0.001$) was reported between 2015 and 2017. In February 2017, the WHO published its first list of antibiotic-resistant "priority pathogens," including Enterobacteriaceae resistant to third-generation cephalosporins (3GCC) and carbapenems, including strains of Escherichia coli and Klebsiella pneumoniae [27].

In a study, it was found that the trimethoprim-sulfamethoxazole combination was active against the majority of Salmonella Enteritidis strains, and the frequency of resistance was 3.51%. Another study showed that the predominant serotypes S. Typhimurium (15.34%) and S. enteritidis (69.84%), have a high resistance rate to the trimethoprim-sulfamethoxazole combination (48.1%), and other authors have also shown high resistance of minor Salmonella to this antibiotic combination [23, 28, 29].

Antimicrobial susceptibility testing showed that E. coli strains were highly resistant to SXT (87.4%) [30].

Sequence Similarity Study

The similarity between the 16S rRNA gene sequences of four reference Salmonella strains (NR074910.1, NC116126.1, OR131043.1, LR991664.1) retrieved from the NCBI website and the two Salmonella strains S-15 and S-36 in our study was examined using GENIEGEN 2 software.

The close kinship of the 16S rDNA of the Salmonella strains in this study with the reference strains belonging to the serovar Salmonella Typhimurium confirmed the serotyping results. Compared with the sequence of strain S-15, the sequences of Salmonella reference strain NR074910.1 is characterized by the highest 16S rDNA similarity percentage (99.52%). Compared with the sequences of strain S-36, the sequence of Salmonella reference strain NR074910.1 is characterized by the highest 16S rDNA similarity percentage (99.2%).

These results indicate that partial 16S rRNA sequencing could provide important phylogenetic characterization between Salmonella. Diagnostic tools for members of the genus Salmonella based on molecular genetics could be improved by 16S rRNA sequencing, which we have demonstrated here. This study demonstrates the value of combining partial 16S rRNA gene sequencing with serotype testing as effective tools for epidemiological tracing of Salmonella at the species level.

Journal of Chemical Health Risks

www.jchr.org

JCHR (2026) 16(1), 1754-1765 | ISSN:2251-6727

6. Conclusion

This study provides a better understanding of the distribution and prevalence of Salmonella in Rabat. The various results of this study necessitate a discussion, in collaboration with poultry farmers, on the importance of enhanced monitoring of farming practices, particularly regarding individual and collective hygiene and biosecurity measures, as well as the control of all incoming materials and equipment.

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