Biofilms and Antibiotic Resistance Determination from Different Immunocompetent Patients with Salmonellosis

Mariam Ayad Abd 1*, Sawsan Q. T. Al-Quhli [1], Mahir Ali Jassim [2]

Abstract

Background: Salmonellosis is an infectious disease due to presence of *Salmonella enterica serovars, S. Typhi* and *S. Typhimurium.* Our research demonstrated the antibiotic susceptibility, biofilm formation and isolation of *Salmonella* from immunocompromised and immunocompetent patients. Methods: Biochemical tests and PCR analysis were employed to determine *Salmonella* isolates (83 isolates out of 217 samples) with the 16S rRNA gene detection. Microtiter plate assay was used to determine the biofilms formation on plastic surfaces. Results: 73.75% *Salmonella* was isolated from clinical samples. Antibiotic susceptibility showed that 85.18% of isolates from immunocompromised patients were sensitive to levofloxacin, and the rest were resistant to multiple antibiotics. 73.1% of *S. Typhi* were sensitive to levofloxacin among immunocompetent patients, and 69.23% were resistant to chloramphenicol. PCR test showed a rapid identification of *Salmonella* serovars. The results demonstrated the biofilm and isolates identification of *Salmonella* significantly. Increased multidrug resistance were observed from the results. Conclusion: The results showed *Salmonella* identification, antibiotic resistance, and molecular diagnosis strategy from clinical samples.

Keywords: Biofilm; Multidrug-resistant; Immunocompromised; Bacterial antibiotic susceptibility testing; Immunocompetent

Significance | This study discussed important information about antibiotic resistance and how harmful *Salmonella* bacteria are in Iraqi patients, helping to plan better public health measures.

*Correspondence. Mariam Ayad Abd, Department of Microbiology, College of Medicine, University of Anbar, 31001, Iraq.
E-mail: mar21m0013@uoanbar.edu.iq

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Introduction

Salmonella enterica stands as a major contributor to community-acquired bacteremia, resulting in elevated hospitalization rates and severe outcomes, particularly among individuals with compromised immune systems and cancer patients (Nyabundi et al., 2017). The susceptibility of cancer patients to *Salmonella* infections is pronounced, often leading to invasive diseases and septic complications (Obaro et al., 2015; Appiah et al., 2021). Conversely, the infection typically manifests as self-limiting diarrheal illness in healthy individuals. Various risk factors, including aging, decreased gastric acid secretion, gastric surgery, antibiotic misuse, and specific medications, contribute to the occurrence of non-typhoidal *Salmonella* infections (Cheesbrough, 2005).

The efficacy of antimicrobial drugs in preventing antibiotic resistance has sparked concerns within the medical community. Farmers are now exploring alternatives to antibiotics, such as probiotics, prebiotics, and organic acids, in an attempt to mitigate the potential development of resistance by microorganisms (Gantois et al., 2009). Despite some regional farmers reporting favorable results, uncertainties persist, highlighting the ongoing challenge of finding effective and sustainable solutions to bacterial resistance.

Non-typhoidal *Salmonella* infections typically present as self-

Author Affiliation.
[1] Department of Microbiology, College of Medicine, University of Anbar, 31001, Iraq.
[2] Internal Medicine Department, College of Medicine, University of Anbar, Iraq.

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-limiting gastroenteritis. However, severe cases leading to bacteremia and extraintestinal infections are prevalent, particularly in vulnerable populations (Fierer, 2022). Timely antibiotic treatment becomes crucial for invasive diseases, emphasizing the critical role of prompt medical intervention (Galanakis et al., 2007; Hussaini et al., 2021). Recently developed formulation and natural agents show promise in preventing antibacterial resistance activity (Abdullah et al. 2024, Hiba et al. 2024, Muntaha et al 2024). The management of multidrug-resistant *Salmonella* strains poses challenges, and preventing biofilm formation emerges as a promising strategy to combat these strains (Lim et al., 2018).

Salmonella infections maintain their significance as a public health concern, particularly impacting vulnerable populations like immunocompromised individuals and cancer patients. The gram-negative bacteria *Salmonella* present unique challenges for those with compromised immune systems, rendering them more susceptible to severe and prolonged illness (CDC, 2019).

Immunocompromised individuals, such as those with HIV/AIDS or organ transplant recipients, face heightened risks due to their weakened immune responses (Geddes et al., 2020). *Salmonella*'s ability to exploit compromised immune defenses can lead to more severe clinical outcomes and increased resistance to antibiotic treatments (Geddes et al., 2020; CDC, 2019).

Cancer patients undergoing treatments like chemotherapy or radiation therapy are particularly vulnerable, as these therapies not only compromise the immune system but may also disrupt the gastrointestinal tract, providing an entry point for *Salmonella* (Shaw et al., 2021). The interplay between weakened immunity and altered gut environments heightens the susceptibility of cancer patients to severe and potentially life-threatening *Salmonella* infections (Shaw et al., 2021).

The repercussions of *Salmonella* infections in vulnerable populations transcend immediate health impacts, encompassing a spectrum of challenges that extend to healthcare systems and resources. The burden on healthcare infrastructure is notably exacerbated by the necessity for hospitalizations, often prolonged treatments, and the potential emergence of long-term complications, as elucidated by Shaw et al. (2021).

Hospitalizations, a common consequence of severe *Salmonella* infections in immunocompromised individuals and cancer patients, strain healthcare resources by requiring specialized care and increased medical attention. The complex medical needs of these patients contribute to extended hospital stays, demanding substantial resources and healthcare personnel dedicated to their treatment and recovery. Prolonged treatments not only intensify the strain on healthcare facilities but also elevate the overall economic burden associated with *Salmonella* infections.

Furthermore, the emergence of antimicrobial resistance amplifies the intricacies of treatment strategies for *Salmonella* infections, as highlighted by Geddes et al. (2020). The evolution of resistance mechanisms poses challenges in effectively combating the bacteria with conventional antibiotics. This resistance not only prolongs the duration of treatment but also necessitates the use of alternative, often more expensive, antimicrobial agents. Consequently, the economic implications of antimicrobial resistance contribute significantly to the escalating healthcare costs associated with managing *Salmonella* infections in vulnerable populations.

Beyond the immediate fiscal impact, the long-term consequences of *Salmonella* infections, particularly in the context of antimicrobial resistance, raise considerable concerns. Geddes et al. (2020) emphasize the need for sustained efforts to understand the evolving landscape of antimicrobial resistance in *Salmonella* strains, as it has the potential to compromise the effectiveness of existing treatments over time. The long-term implications extend to the development of novel therapeutic approaches, the establishment of stringent infection control measures, and the implementation of surveillance strategies to curb the spread of resistant strains.

Addressing the significance of *Salmonella* infections in immunocompromised individuals and cancer patients necessitates a comprehensive approach. Collaborative efforts among healthcare professionals, researchers, and policymakers are essential to developing targeted strategies for the management and prevention of *Salmonella* infections in these vulnerable populations. Emphasizing preventive measures, stringent food safety practices, and tailored medical interventions will contribute to mitigating the associated health and economic burdens (CDC, 2019; Geddes et al., 2020; Shaw et al., 2021).

Biofilms, complex communities of microorganisms encased in a protective extracellular matrix, emerge as crucial contributors to the challenges posed by multidrug-resistant (MDR) *Salmonella* infections. These biofilms create a resilient environment that shields *Salmonella* from the effects of conventional antibiotics, rendering eradication efforts significantly more difficult (Igomu, 2020; Tang et al., 2006; S. M. Kamrul et al. 2020). This intricate interplay between *Salmonella*, antibiotic resistance, and biofilm formation underscores the need for a nuanced understanding of the factors influencing infection severity and treatment efficacy.

Understanding the dynamics of biofilm formation is essential for devising effective strategies to manage MDR *Salmonella* infections. Igomu (2020) highlights the intricate nature of biofilms in providing a protective shield, making conventional antibiotic treatments less effective. Tang et al. (2006) further emphasizes the role of biofilm in promoting bacterial persistence and resilience, complicating the task of eradicating *Salmonella* from both clinical and agricultural environments.

This study aims to examining the link between Multidrug-Resistant *Salmonella* and Biofilm Formation in Immunocompromised vs. Immunocompetent Patients within a specific geographical area.

Materials and methods

Patients

The study comprises 217 male and female patients aged 1 to 60 years, presenting clinical suspicion of S. enterica infection with gastroenteritis. Over the period from October 2022 to October 2023, participants were sourced from Al-Ramadi Teaching Hospital Laboratory, Al-Ramadi Teaching for Children and Maternity Hospital Laboratory, Anbar Cancer Center, and Ramadi Special Private Clinics.

Types of Antibacterial Discs and Concentration

The Antibiotic discs used in the present study were, Amikacin (AK) at 30 µg, Amoxycillin (AX) at 25 µg, Amoxicillin clavulanic acid (AUG) at 30 µg, Levofloxacin (Lev) at 5 µg, Gentamicin (CN) at 10 µg, Ceftriaxone (CTR) at 30 µg, Cefotaxime (CTX) at 30 µg, Cefixime (Cf) at 30 µg, Ciprofloxacin (CIP) at 5 µg, and Chloramphenicol (C) at 30 µg.

Polymerase Chain reaction materials

Suspected typhoid fever patients met the criteria after examination. Blood (5-10ml) and stool samples were collected from Al-Ramadi Teaching Hospital and private laboratories. Blood samples were incubated at 37°C for three days and directly inoculated onto MacConkey, Blood, and XLD agar, incubated for 18-24 hours (Igomu, 2020). Stool samples from suspected *S. enterica* serovar Typhimurium and *S. enterica* serovar Typhi cases were gathered following WHO guidelines (Tang et al., 2006). Bacterial isolates were tested for gram sustainability using Gram staining, which involves applying a crystal violet stain, adding mordant, decolonizing with ethanol or acetone, and counterstaining with safranin.

Biochemical tests

Various biochemical tests assessed bacterial traits. The Oxidase Test used reagent-soaked paper to show a purple reaction for oxidase production within 20-30 seconds. Catalase Test indicated enzyme presence through bubble formation with hydrogen peroxide. Kligler Iron Agar (KIA) detected acid, gas, and H2S production via color shifts, bubbles, and black residue. Indole Production Test in Peptone broth revealed tryptophanase activity with a deep red ring. Simmon's citrate agar shifted from green to blue, signifying sodium citrate utilization. Urease Production Test on urea agar detected ammonia and carbon dioxide, displaying pink for positive and yellow for negative results (Tang et al., 2006).

Identification of *Salmonella entrica* Vitek-2 System

Bacterial isolates interact with the VITEK-2 Identification Cards in a biochemical response. These isolates were streaked on MacConkey agar plates and suspended in a solution matching McFarland 0.5 standard. The suspensions were loaded into the Gram-Negative and Gram-Positive VITEK-2 ID cards, followed by manual loading into the VITEK-2 system per manufacturer instructions (Akter & Shamimuzzaman, 2023).

The GN/AST system utilized ASTN084 and ASTN093 cards for MIC determination. The ASTN084 card specifically targeted extended-spectrum beta-lactamase (ESBL) antibiotics. To interpret MIC results, they were linked to organism identification and categorized according to CLSI guidelines (CLSI, 2017).

Antibiotic susceptibility test

The test followed the Kirby-Bauer (disk diffusion) technique using Muller-Hinton agar and various commercially supplied single antibiotic discs as per the Clinical Laboratory Standards Institute . Antibiotic susceptibility and resistance were assessed based on strain growth zone diameter, utilizing a set of 10 antibiotic discs for this study (CLSI, 2017). The process involved selecting three to five well-isolated colonies of the organism and suspending them in 5ml of sterile saline. Turbidity was matched to the McFarland standard (VITEK2 McFarland). After swabbing the medium's surface, excess inoculum was removed. The inoculum dried before placing ten discs on a 15 cm Muller Hinton plate per patient, spaced adequately. Following overnight incubation, zone diameters around the discs were measured in millimeters. Interpretation was based on zone diameters categorized as sensitive, intermediate, or resistant, referencing the CLSI's antibiotic susceptibility testing chart (CLSI, 2017).

Quantitative Biofilm Assay

Following Halim et al. (2018), the Microtiter plate assay involved culturing bacterial cells in TSB broth, diluting them to 0.5 McFarland standards, and incubating them in a 96-well plate at 36±1 °C for 24 hours. After removing free-floating bacteria, adherent cells were fixed with methanol and stained with crystal violet. Following washing and dye solubilization with ethanol, absorbance was measured at 490 nm using an ELISA reader. Adhesion strength was assessed based on absorbance values (none, weak, moderate, strong). Halim et al. (2018) Conducted the test three times, averaging the results and interpreting absorbance values to determine the biofilm's extent (Halim et al., 2018).

Molecular determination

Genomic DNA was extracted from *S. enteric serovarTyphi*and *S. entercaserovarTyphimurium*isolates using Genesis Genomic DNA Purification Kit (Add bio) and produced as directed by the company. To analyze Gram-negative bacterial cells, centrifuge overnight, resuspend in Lysis Solution, add Proteinase K solution, and incubate at 56°C. For RNA-free genomic DNA, add RNase A Solution and incubate. Transfer lysate to spin column, centrifuge, wash, and elute genomic DNA. Finally, add Elution Solution and centrifuge for 1 min.

DNA concentration and purity are determined using a UV spectrophotometer (Geneo), measuring optical density at 260 and 280 nm. DNA concentration is calculated by multiplying

absorbance at 260 nm by conversion and dilution factors. Total DNA yield is derived from DNA concentration multiplied by the final purified sample volume. DNA purity is determined by the ratio of 260 and 280 nm readings, ideally between 1.4 and 2.0 (Sambrook et al., 1989).

The 1.0% Agarose gel prep involved dissolving 1.0 gm of Agarose in 100ml of (1x) TBE buffer (pH= 8) for pre-PCR and 1.5 gm for post-PCR. After cooling, 0.5 red safe dye was added, wells formed, and the gel was left to solidify for 15-20 mins at room temp. Once set, the comb was removed, and the gel was placed in the electrophoresis chamber and filled with TBE buffer to cover the gel. DNA samples (10 µl) were mixed with loading dye (2µl) and then loaded into the wells in agarose gel. Electric current was allowed at 70 volts for 90 minutes. A UV transilluminator was used to observe the DNA bands.

Molecular Identification of *S. enterica serovar* Typhi and *S. entericaserovar* Typhimurium by PCR techniques

Primers sourced from Macrogen Company in Korea (Table 1, 2) were prepared per the manufacturer's instructions. Lyophilized primers were reconstituted using deionized distilled water (ddH2O) to achieve various concentrations (Pico moles/microliter). Stock solutions were obtained by vortexing and centrifuging, then stored at -20 °C. To create working solutions, 10 µl from the stock was mixed with 90 µl ddH2O in a separate tube, vortexed, and held at -20 °C for use. A DNA ladder (100bp) obtained from Biolab New England company at a concentration of 50µg/ml (100 – 1517) was used to gauge the sizes of DNA fragments post polymerase chain reaction. The ladder was visualized using ethidium bromide staining (5µg/ml) in a 1.5% agarose gel.

Statistical Analysis

All data were analyzed using the Chi-square (Cross tabulation) or Mann-Whitney test. All graphics of this study (dot chart, bar chart, or scatter diagram) have been achieved by Microsoft Excel ver.2020.

Results

***Salmonella enterica* Isolation and Identification**

The first identification of *S. enterica serovar Typhi* and *S. enterica serovar Typhimurium* isolates has been based on morphological, biochemical, and microscopical studies. Microscopically, the bacteria were seen as Gram-negative, small rods in shape arranged in single or pairs. *S. enterica serovar Typhi* and *S. enterica serovar Typhimurium* were grown on various media at 37°C for 18-24 hours. Notable characteristics included small, smooth, red-hued colonies with a black core on XLD agar; non-lactose fermenting, colorless colonies on MAC; and non-hemolytic, white colonies on blood agar. These findings confirmed the presence of *S. typhi* and *S. typhimurium*, consistent with Salim et al. (2022). XLD agar's selective nature facilitated *Salmonella* growth while inhibiting E. coli. VITEK® 2 compact authenticated all *Salmonella* isolates.

The TSI, Sugars, Oxidase, Indole, Ureases, and Simmons citrate assays indicated the biochemical results of *Salmonella* isolates. In the TSI slants test, the slant and butt turned AKL/ACID red and yellow, suggesting non-fermentation of glucose on the slant and acid generation with H2S in the bottom. Further tests of *Salmonella* isolates yielded negative results for oxidase, indole generation, urease generation, and citrate use. The identification of *Salmonella* isolates using the VITEK-2 GN ID Cards System comprised various biochemical assays. Total positive culture samples and Vitek of *Salmonella* isolates = 83/217 (38.4%).

Out of 80 patients, 43 (54.2%) were male, and 37 (45.8%) were female. Therefore, males were estimated to be infected at a higher rate than females. This result is compatible with the results of the studies in the Al-Musaib and Diyala districts. That could be because most males were out-doored and could be seen as food-eating and handling or contact with other patients from this perspective. The patients' age rate is from (5-60) years old; the patients' ages are distributed in Table 3, 4. S. enterica isolates exhibited higher infection rates in the 5-10 (28.91%) and 11-20 (21.81%) age groups, potentially linked to increased mobility and consumption of unhygienic food and water in school and college settings. This aligns with previous studies by Sur et al. (2007), and Al-Kraety (2017).

The lowest incidence was among the (21-30) and (51- 60) age groups, 10.8% and 8.4%, respectively related to frequent boosting of immunity. Positive cultures of Salmonella enterica serovar typhi and Salmonella enterica serovar typhimurium samples revealed a significant difference with a P-value of 0.005.

The results match prior studies (Al-Kraety & Al-Ammar, 2017). While we surpassed AL-Taie's (2009) findings, our rates were lower than Babylon's 8.0% positive *Salmonella* cases (Halim et al., 2018). In contrast, Seraj (2018) reported a 57.0% incidence in Al-Nasiriyah, Southern Iraq, possibly due to differing hygiene and feeding practices. Studies link higher *Salmonella* rates to immunodeficiency.

Confirmation of *salmonella* isolates by PCR amplification of 16srRNA gene

PCR techniques targeting the 16srRNA genes of S. enterica serovar typhi and *S. enterica* serovar typhimurium were employed for sample analysis. Figure (1) illustrates the PCR method confirming *S. enterica* serovar Typhimurium identification via a 425 bp product within the 16srRNA gene. Using Vitek 2compact methods, 63 samples contained this specific gene. This study underscores the utility of 16S rRNA genes in identifying the *Salmonella* genus and species, consistent with findings by (Abdelaziz, 2023).

Biofilm formation of *S. enterica* by microplate titer assay

The results of the microtiter experiment in the current investigation indicate that the isolates of *Salmonella enterica* demonstrated a significant potential for producing biofilm on plastic surfaces.

According to the analysis, the isolates were categorized into different groups based on their biofilm formation capacities.Out of the 83 clinical bacterial isolates of *S. enterica* tested, biofilm

Table 1. Primers used in this study.

Primers		Sequences	product	annealing
16srRNA	F	CGGACGGGTGAGTAATGTCT	406	58
	R	GTTAGCCGGTGCTTCTTCTG		
adaA	F	TCGGGTGAGTCCTGAAAAAC	173	58
	R	ACGATCATCAGGAGGGTCAG		
csgD	F	ATTAACGGCGTGTTTTACGC	228	57
	R	CGACCTCGCGATTTCATTAT		
gcpA	F	CCAGCTCGGTTACAAGTCGT	203	58
	R	GATCGGCTATCCAGTTCAGG		
recA	F	GGGAACCTGAAACAGTCCAA	188	58
	R	GTTTCGCTACCCACGACATT		

Table 2. Mean of OD value and biofilm formation (Halim et al., 2018).

Mean OD value	Biofilm formation
0.15<	No biofilm
0.15-0.24	Weak biofilm
0.25-0.39	Moderate
0.4	Strong

Table 3. Distribution of the patient according to gender and age groups of *S. enterica* infection

Age group	Total NO. (%)	NO. of Male (%)	NO. of Female (%)
5-10	24 (28.91)	15(62.5)	9(37.5)
11-20	18 (21.81)	11 (61.1)	7(38.88)
21-30	9 (10.8)	4(44.4)	5(55.6)
31-40	11 (13.25)	5 (45.5)	6(54.5)
41-50	14 (16.87)	6 (42.8)	8(57.1)
51-60	7 (8.4)	4 (57.1)	3(42.9)
Total	83 (100%)	45(54.2%)	38(45.8%)

Table 4. Isolation and identification of *S. enterica typhi* and *S. enterica Typhimurium*

Total samples: 217(100) %			
Total samples of positive culture and Vitek of Salmonella isolates= 83(38.4) %			
Type samples	Total No. of *Salmonella* isolates %	Total No. of *S. typhi*%	Total No. of *S. Typhimurium* %
Stool	73	8	65
Blood	10	10	0
	83(100%)	(21.7 %)	65(78.31%)

Table 5. Distribution of the *S.entrica* patient according to type case

Type of case	Immunocompromised patients	Immunocompetent patients	Total
Number of samples	89	128	217
Positive	48(57.8%)	35(42,16%)	83 (38.4%)
Negative	41(30.59%)	93(69.4%)	134(61.75%)
	89(100%)	128(100%)	

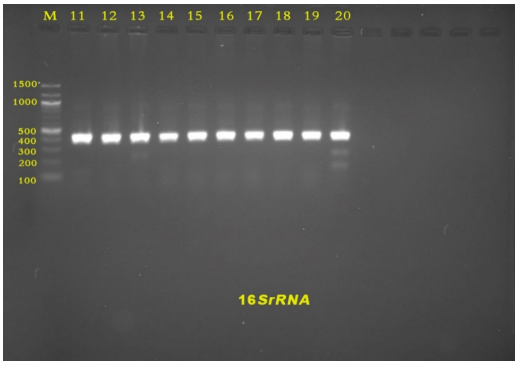

Figure 1. Molecular identification of S. entrica isolates by 16srRNA gene with product 406, DNA Ladder(100bp), agarose conc.(1.5%) with Red Safe stain and electric current at 60 V for 85 min.

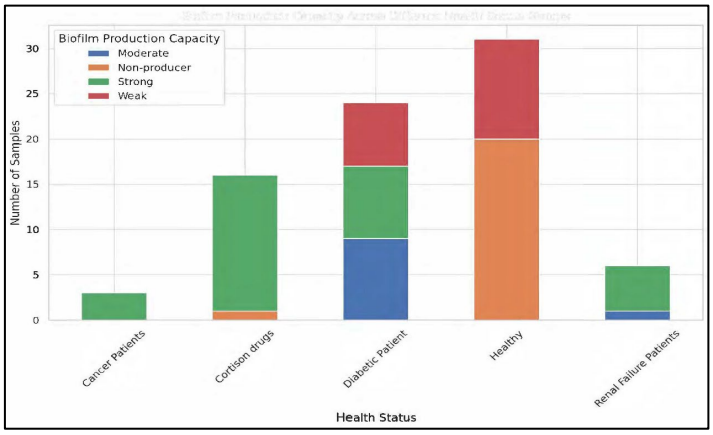

Figure 2. Biofilm Production Capacity Across Different Groups

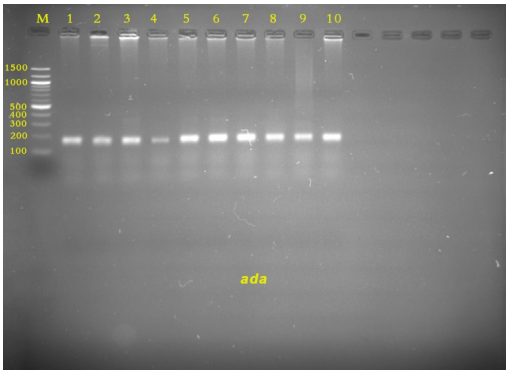

Figure 3. Amplification products of adrA gene of S.enterica by PCR with product 173bp, DNA Ladder(100bp), agarose conc.(1.5%) and red safe

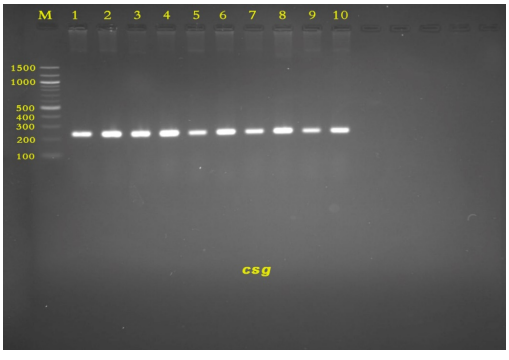

Figure 4. Amplification products of CsgD gene of S.enterica by PCR with product 203bp, DNA Ladder(100bp), agarose conc.(1.5%) and Red safe stain; electric current at 60 volts for 85 mints.

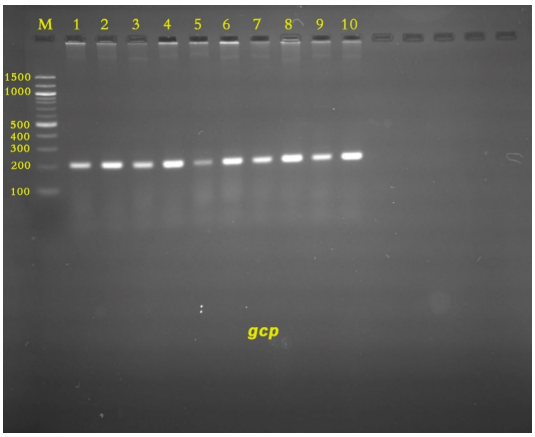

Figure 5. Amplification products of GCP gene of *S.enterica* by PCR with product 173bp, DNA Ladder(100bp), agarose conc.(1.5%) and red safe stain; electric current at 60 volts for 85 mint.

Table 6. Comparison of antibiotic susceptibility in immunocompromised (ICD) and immunocompetent (ICT) cases for various antibiotics tested against S. enterica.

Antibiotics	**Sensitive (%) ICD**	**Intermediate(%) ICD**	**Resistant (%) ICD**	**Sensitive (%) ICT**	**Intermediate(%) ICT**	**Resistant (%) ICT**
Amoxicillin	0.00	0.0%	100.0	19.23	23.1%	57.70
Amikacin	57.60	7.4%	35.1	61.50	26.9%	11.50
Cefixime	0.00	0.0%	100.0	40.60	31.3%	28.10
Levofloxacin	85.18	11.1%	3.7	73.10	19.23%	7.60
Ampicillin	0.00	0.0%	100.0	19.23	26.92%	53.80
Gentamycin	57.40	18.5%	24.1	65.50	30.8%	3.80
Chloramphenicol	0.00	0.0%	100.0	15.38	15.38%	69.23
Ceftriaxone	0.00	0.0%	100.0	23.07	19.23%	57.70
Cefotaxime	0.00	0.0%	100.0	11.50	42.3%	46.20
Amoxicillin/Clavulanic	55.60	24.1%	20.3	55.60	23.1%	20.30

production was detected in 61 isolates (73.75%), indicating a high capacity to form biofilm on plastic surfaces. The remaining 22 isolates (26.25%) were classified as non-producer biofilms with OD values below 0.15 **(Figure 2**).

Among the biofilm-producing *Salmonella* isolates, 35.59% showed strong biofilm formation, indicating robust adherence to microplate plastic surfaces. Moderate and weak biofilm formation was observed in 35.59% and 28.8% of isolates, respectively. This demonstrates a high capability of *Salmonella* to form biofilms on plastic, with implications for public health and food safety.

Detection of the virulence gene in the *S. entries* by PCR(adrA, csgD, and gcpA)

In the present study, a positive result was that 70% of isolates of *S . typhi* and *S. typhimurium* produced 173 bp DNA fragments specific for the adrA gene.

This study showed that all isolates of *S. typhi* and *S. Typhimurium* produced 203bp DNA fragments specific for the CsgD gene, and these isolates had positive results. This result agrees with (Seixas et al.,2014) Figure 3, 4, 5.

This study also showed that more than 50% of isolates of *S. typhi* and *S. Typhimurium* produced 173bp DNA fragments specific for the GcpD gene, and these isolates had positive results(Figure 5).

Antibiotic susceptibility

The most common and basic antibiotic susceptibility testing techniques, disc-diffusion, and micro broth dilution procedures were included (Patel et al., 2011). As antimicrobials, all of the isolates in this investigation are resistant to four or more types of antibiotics.

Distribution of antibiotic Testing of *S. entries*by disk diffusion method in immunocompromised case

Antibiotic susceptibility of S. entrica isolates was tested using ten antibiotic discs according to CLSI guidelines. Results (Table 6) showed that 46/54 isolates (85.18%) were sensitive to Levofloxacin. All isolates (100%) were resistant to ampicillin, chloramphenicol, cefotaxime, ceftriaxone and cefixime. 30/54 isolates (55.6%) were resistant to amoxicillin-clavulanic acid. 29/54 isolates (53.7%) were sensitive to gentamicin. 30/54 isolates (55.6%) were susceptible to amikacin. 31/54 isolates (57.4%) were susceptible to ciprofloxacin. This suggests that *S.* entrica biofilm formation may be reduced by certain antibiotics.

Notably, no resistances were observed among the isolates to Amikacin, Gentamycin, and Ciprofloxacin. Levofloxacin demonstrated effectiveness against Gram-negative biofilms, hindering biofilm formation (Anderl et al., 2000). Conversely, most *S. Typhimurium* isolates exhibited resistance to antibiotics such as Ceftriaxone, amoxicillin, ampicillin, cephalexin, amoxicillin-clavulanic acid, and cefixime, adapting to combat antibiotics (MacKenzie et al., 2017). *Salmonella* exposure facilitated biofilm formation through increased polysaccharide synthesis, where some antibiotics hinder biofilm production without killing bacteria (Babu et al., 2015).

Distribution of antibiotic Testing of *S. enterica* by disk diffusion method in the immunocompetent case

Among S. typhi isolates, sensitivity was observed in 73.1% to Levofloxacin, 61.5% to Amikacin and ciprofloxacin, and 65.5% to Gentamycin. Conversely, resistance was prevalent, with 69.23% resistant to Chloramphenicol, 84.6% to Amoxicillin, 57.7% to ceftriaxone, and 46.2% to cefotaxime. Other resistant and intermediate results are detailed in Table 6. The differences in antibiotic sensitivity, intermediates, and resistance were statistically significant (P-value < 0.005), aligning with findings by Israa (Israa, 2020).

The prevalence of antibiotic resistance among the isolates in this study might stem from unrestricted access to antibiotics without prescriptions in clinics, private pharmacies, and markets. This widespread availability may have exerted selection pressure on bacterial populations, contributing to high resistance rates. Various factors, such as indiscriminate use and control of antimicrobials, have also been linked to resistance development in gastrointestinal infections (Dakorah, 2014).

Discussion

Accurately identifying bacterial isolates is fundamental to understanding the epidemiology and pathogenicity of microbial agents. In this study, we followed a series of procedures to identify and confirm the presence of two crucial *Salmonella serovars, S. enterica serovar Typhi* and *S. enterica serovar Typhimurium*. These procedures included culture-based methods, microscopic examination, biochemical tests, molecular identification, assessment of biofilm formation, and antibiotic susceptibility testing.

Biochemical tests further corroborated the identification of *Salmonella* isolates. These tests provided valuable insights into the characteristics of the isolates and their ability to metabolize specific substrates.

The epidemiological aspect of our study revealed exciting trends in *Salmonella* infections among different age groups. Notably, the age groups of 5-10 years and 11-20 years exhibited the highest infection rates, possibly due to increased mobility and unhygienic food and water consumption in school and college settings. These findings align with previous studies conducted by Sur et al. (2007) and are consistent with the observations of Al-Kraety (2017).

S. enterica serovar Typhimurium emerged as the most predominant serovar among the patients, consistent with the results reported by Korah, M. P. (2014). Interestingly, the lowest incidence was observed in the age groups of 21-30 and 51-60, possibly attributed

to a more robust immune response in these age groups, as suggested by Ja 'afar et al. (2013).

The prevalence of *Salmonella* isolates in our study, at 38.4%, is in line with the findings of Al-Kraety (2017) but exceeds the isolation percentage reported by AL-Taie (2009) in a different region. The disparities in *Salmonella* infection rates can be attributed to varying dietary habits and hygiene practices in other populations.

Immunodeficiency diseases have been associated with increased susceptibility to *Salmonella* infections, as highlighted by Bokhari et al (2016). These diseases compromise immune function, impair phagocytic responses and compromise gut barrier integrity.

Furthermore, reductions in antimicrobial peptide production further exacerbate susceptibility to *Salmonella* infections.

Using molecular techniques such as PCR, targeting the 16S rRNA gene provided a reliable method for confirming the identification of *S. Typhimurium* and *S. Typhi* isolates, consistent with the findings of Abdelaziz (Abdelaziz, 2023).

Detection of the virulence genein the *S.* entries (adrA, csgD, and gcpA)allows researchers to study its sequence and expression and explore its role in *Salmonella* pathogenesis and the mechanisms by which it interacts with the host.The adrA gene in *Salmonella enterica* plays a role in bacterial adhesion and invasion by contributing to the pathogenicity of the organism. The CsgD gene encodes a transcriptional regulator known as CsgD (Curli-specific regulator D). CsgD is a key regulator of curli fimbriae and cellulose production in *Salmonella enterica* andthe GcpD gene is critical for activating cellulose synthesis and biofilm formation (Chen et al., 2021).

Understanding the regulatory functions of these genes, particularly adrA, CsgD and GcpD, sheds light on biofilm dynamics and holds promise for targeted intervention strategies against *Salmonella* infections.These findings collectively accentuate the significance of the adrA, CsgD, and GcpD genes in the pathogenesis and biofilm formation of *Salmonella* enterica, laying the groundwork for deeper investigations into targeted therapeutic interventions or preventive strategies aimed at mitigating *Salmonella* infections.

Biofilm formation is a significant virulence factor for many pathogens, including *Salmonella*. Our study demonstrated a substantial potential for biofilm formation by *Salmonella* enterica isolates on plastic surfaces. This observation is crucial, as biofilm formation can contribute to the persistence of *Salmonella* in various environments, posing a public health risk.

Antibiotic susceptibility testing revealed concerning levels of resistance among the *Salmonella* isolates. Most isolates exhibited resistance to multiple antibiotics. This high level of antibiotic resistance may be linked to the overuse and inappropriate dispensing of antibiotics without prescription, as antibiotics are readily accessible in clinics, private pharmacies, and markets. Similar findings were reported by (Israa, 2020), emphasizing the need for stringent antibiotic use policies and surveillance.

Conclusion

In conclusion, this study provided important insights into the antibiotic resistance patterns and virulence characteristics of *Salmonella enterica* isolates in Iraqi patients. A high prevalence of multidrug-resistant strains was observed among isolates from immunocompromised and immunocompetent patients. Most isolates resisted multiple first-line antimicrobial agents used to treat salmonellosis in the region. Additionally, over 70% of isolates demonstrated a strong capacity for biofilm formation on abiotic surfaces. The ability of *Salmonella* to form biofilms poses challenges for antimicrobial therapy and environmental decontamination.

Author contribution

M.A.A. designed, analyzed, performed experiments and write the article, S.Q.T.A.Q. analyzed data, wrote the article, M.A.J. conceptualized, data analyzed, and write the article.

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Competing financial interests

The authors have no conflict of interest.

References

Abbas, K. K., AL-Wattar, W. M. A., Hasan, S., Kasim, H., & Jasim, A. A. (2017). The incidence of Shigella and Salmonella in the stool of pediatric patients. Iraqi Journal of Public Health, 1(3).

Abdulah Y. Al-Mahdi, Alabed Ali A. Alabed, Mohammed Faez Baobaid et al., (2024). Antibacterial Activity of Herbal Essential Oils against Gram-positive and Gram-negative Bacteria with a Potential for Multidrug Resistance, Journal of Angiotherapy, 8(2), 1-7, 9517.

Akter, F., & Shamimuzzaman, M. (2023). Detection of invA Gene by PCR and Identification with Susceptibility Pattern Evaluation by VITEK 2 of Salmonella Isolates.

AL-Khafaji, J. K. T., AL-Yasari, H. F., & AL-Taei, M. H. (2006). Prevalence of typhoid fever among pediatric patients at Al-Musaib District. Medical Journal of Babylon, 3(1-2).

AL-Kraety, I. A. A., & Al-Ammar, M. (2017). relation of class1 integron gene with multidrug resistance salmonella typi isolates. Pak. J. Biotechnol. Vol, 14(4), 537-541.

AL-Taie, H. H. I. (2009). Prevalence rate of Salmonella in Babylon province. The Iraqi Journal of Veterinary Medicine, 33(2), 152-157.

Anderl, J. N., Franklin, M. J., & Stewart, P. S. (2000). Role of antibiotic penetration limitation in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin. Antimicrobial agents and chemotherapy, 44(7), 1818-1824.

Appiah, G. D., Mpimbaza, A., Lamorde, M., Freeman, M., Kajumbula, H., Salah, Z., Kugeler, K., Mikoleit, M., White, P. B., & Kapisi, J. (2021). Salmonella bloodstream infections in hospitalized children with acute febrile illness—Uganda, 2016–2019. The American Journal of Tropical Medicine and Hygiene, 105(1), 37.

Appiah, G. D., Mpimbaza, A., Lamorde, M., Freeman, M., Kajumbula, H., Salah, Z., Kugeler, K., Mikoleit, M., White, P. B., & Kapisi, J. (2021). Salmonella bloodstream infections in hospitalized children with acute febrile illness—Uganda, 2016–2019. The American Journal of Tropical Medicine and Hygiene, 105(1), 37.

Babu, D., Kalpana, K., & Juneja, V. K. (2015). Emergence of Drug-Resistant Pathogens. In Foodborne Pathogens and Food Safety (pp. 131-142). CRC Press.

Bukhari, E., Alaklobi, F., Bakheet, H., Alrabiaah, A., Alotibi, F., Aljobair, F., Alshamrani, M., Alaki, E., Alnahdi, F., & Alodyani, A. (2016). Disseminated bacille Calmette-Guérin disease in Saudi children: clinical profile, microbiology, immunology evaluation and outcome. European Review for Medical & Pharmacological Sciences, 20(17).

CDC. (2019). Salmonella. Retrieved from https://www.cdc.gov/salmonella/index.html

Cheesbrough, M. (2005). District laboratory practice in tropical countries, part 2. Cambridge university press.

Cheesbrough, M. (2005). District laboratory practice in tropical countries, part 2. Cambridge university press.

Chen, S., Feng, Z., Sun, H., Zhang, R., Qin, T., & Peng, D. (2021). Biofilm-formation-related Genes csgD and bcsA promote the vertical transmission of Salmonella Enteritidis in chicken. Frontiers in Veterinary Science, 7, 625049.

CLSI : Clinical and Laboratory Standards Institute. Performance (2017). (27 ed.).

Dakorah, M. P. (2014). Prevalence of salmonella infections in patients attending St. Dominic hospital,(Akwatia)-Eastern Region

Ed-Dra, A., Filali, F. R., Khayi, S., Oulghazi, S., Bouchrif, B., El Allaoui, A., Ouhmidou, B., & Moumni, M. (2019). Antimicrobial resistance, virulence genes, and genetic diversity of Salmonella enterica isolated from sausages. European Journal of Microbiology and Immunology, 9(2), 56-61.

Fierer, J. (2022). Invasive non-typhoidal Salmonella (iNTS) infections. Clinical Infectious Diseases, 75(4), 732-738.

Flayyih, I. A. A. (2017). Molecular characterization of MDR Salmonella sp. Isolate from typhoid patients in Al. Najaf Al. Ashraf province-IRAQ University of Kufa].

Galanakis, E., Bitsori, M., Maraki, S., Giannakopoulou, C., Samonis, G., & Tselentis, Y. (2007). Invasive non-typhoidal salmonellosis in immunocompetent infants and children. International Journal of Infectious Diseases, 11(1), 36-39.

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Gast, R., Humphrey, T. J., & Van Immerseel, F. (2009). Mechanisms of egg contamination by Salmonella Enteritidis. FEMS microbiology reviews, 33(4), 718-738.

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Gast, R., Humphrey, T. J., & Van Immerseel, F. (2009). Mechanisms of egg contamination by Salmonella Enteritidis. FEMS microbiology reviews, 33(4), 718-738.

Geddes, E., Collins, M., Abidi, S. S. R., Lees, J., Serrano, K. J., Gray, B., & Salmond, G. P. C. (2020). A comprehensive study of antibacterial activity of multi-metal doped SnO2 nanoparticles. Frontiers in Microbiology, 11, 348.

Halim, R. M. A., Kassem, N. N., & Mahmoud, B. S. (2018). Detection of biofilm producing staphylococci among different clinical isolates and its relation to methicillin susceptibility. Open access Macedonian journal of medical sciences, 6(8), 1335.

Hiba Ahmed Jawade, Zahraa Yosif Motaweq, Hawraa Dheyaa Rasool et al. (2024). Study of Antibiotic Resistance in ESKAPE Bacteria Using β-lactamase and ESBL Genes, Journal of Angiotherapy, 8(3), 1-8, 9618.

Hussaini I.M., Suleiman A.B. et al. (2021). Molecular Detection and Characterization of Carbapenemases Among Carbapenem-Resistant Escherichia Coli And Klebsiella Pneumoniae Isolated from Urine, Journal of Primeasia, 2(1), 1-6, 20212

Igomu, E. (2020). Salmonella Kentucky: prevalence and challenges in Nigeria and the Africa continent. African Journal of Clinical and Experimental Microbiology, 21(4), 272-283.

Igomu, E. (2020). Salmonella Kentucky: prevalence and challenges in Nigeria and the Africa continent. African Journal of Clinical and Experimental Microbiology, 21(4), 272-283.

Le Thi Phuong, T., Rattanavong, S., Vongsouvath, M., Davong, V., Phu Huong Lan, N., Campbell, J. I., Darton, T. C., Thwaites, G. E., Newton, P. N., & Dance, D. A. (2017). Non-typhoidal Salmonella serovars associated with invasive and non-invasive disease in the Lao People's Democratic Republic. Transactions of the Royal Society of Tropical Medicine and Hygiene, 111(9), 418-424.

Lim, S. H., Methé, B. A., Knoll, B. M., Morris, A., & Obaro, S. K. (2018). Invasive non-typhoidal Salmonella in sickle cell disease in Africa: is increased gut permeability the missing link? Journal of translational medicine, 16, 1-7.

MacKenzie, K. D., Palmer, M. B., Köster, W. L., & White, A. P. (2017). Examining the link between biofilm formation and the ability of pathogenic Salmonella strains to colonize multiple host species. Frontiers in Veterinary Science, 4, 138.

Muntaha R. Ibraheem, Dhafar N. Al-Ugaili. (2024). Nanoparticle-Mediated Plasmid Curing in Combating Antibiotic Resistance in Pathogenic Bacteria, Journal of Angiotherapy, 8(3), 1-8, 9495.

Nyabundi, D., Onkoba, N., Kimathi, R., Nyachieo, A., Juma, G., Kinyanjui, P., & Kamau, J. (2017). Molecular characterization and antibiotic resistance profiles of Salmonella isolated from fecal matter of domestic animals and animal products in Nairobi. Tropical Diseases, Travel Medicine and Vaccines, 3, 1-7.

Obaro, S. K., Hassan-Hanga, F., Olateju, E. K., Umoru, D., Lawson, L., Olanipekun, G., Ibrahim, S., Munir, H., Ihesiolor, G., & Maduekwe, A. (2015). Salmonella bacteremia among children in central and northwest Nigeria, 2008–2015. Clinical Infectious Diseases, 61(suppl_4), S325-S331.

Patel, J. B., Tenover, F. C., Turnidge, J. D., & Jorgensen, J. H. (2011). Susceptibility test methods: dilution and disk diffusion methods. Manual of clinical microbiology, 1122-1143.

SALIM, L., & MOTAWEQ, Z. Y. (2022). Phenotypically and genotypically estimation of virulence factors in Salmonella serovar typhi isolated from patients with enteric fever in Al-Najaf, Iraq. Nusantara Bioscience, 14(1).

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold spring harbor laboratory press.

Seixas, R., Machado, J., Bernardo, F., Vilela, C., & Oliveira, M. (2014). Biofilm formation by Salmonella enterica serovar 1, 4,[5], 12: i:-Portuguese isolates: a phenotypic, genotypic, and socio-geographic analysis. Current Microbiology, 68, 670-677.

Shaw, K. S., Cruz-Cano, R., Jiang, C., Baker, K., Dreyfuss, J. M., & M'ikanatha, N. M. (2021). Epidemiology and trends of salmonellosis in the USA, 1996–2016. Epidemiology and Infection, 149, e8.

S. M. Kamrul Islam, Maruf Abony et al. (2020). Prevalence of Multi-Drug Resistant (MDR) Pseudomonas sp. among patients attending in tertiary care hospital, Dhaka city, Bangladesh, Journal of Primesia University, 1(1), 1-8, 560011

Song, W., Shan, Q., Qiu, Y., Lin, X., Zhu, C., Zhuo, Z., Wang, C., Tong, J., Li, R., & Wan, C. (2022). Clinical profiles and antimicrobial resistance patterns of invasive Salmonella infections in children in China. European Journal of Clinical Microbiology & Infectious Diseases, 41(10), 1215-1225.

Sur, D., Ali, M., Von Seidlein, L., Manna, B., Deen, J. L., Acosta, C. J., Clemens, J. D., & Bhattacharya, S. K. (2007). Comparisons of predictors for typhoid and paratyphoid fever in Kolkata, India. BMC public health, 7(1), 1-10.

Tang, Y.-W., Stratton, C. W., & Aslanzadeh, J. (2006). Biochemical profile-based microbial identification systems. Advanced techniques in diagnostic microbiology, 84-116.

Ugboko, H., & De, N. (2014). Mechanisms of Antibiotic resistance in Salmonella typhi. Int J Curr Microbiol App Sci, 3(12), 461-476.