Volume 3, Issue 3, 2025

Frontier in Medical & Health Research

ISSN: (e) 3007-1607 (p) 3007-1593

ISOLATION, BIOCHEMICAL CHARACTERIZATION AND MOLECULAR TYPING OF SALMONELLA ENTERICA FROM FRESH MEAT. PUBLIC HEALTH PERSPECTIVE

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DOI: https://doi.org/10.5281/zenodo.15425367

Keywords
Salmonella enterica, fresh meat, biochemical characterization, 16S rRNA sequencing, public health.

Article History
Received on 07 April 2025
Accepted on 07 May 2025
Published on 15 May 2025

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Abstract

Salmonella enterica is associated with foodborne infections throughout the world, making its presence in fresh meat a serious public health concern. To determine the Potential risk to human health, this study aimed to Isolate, biochemically Characterize, and molecularly type S. enterica strains from fresh meat samples. Four fresh meat samples of pork, chicken, and beef were Collected from different markets and examined microbiologically. Isolation was carried out using selective enrichment and differential media, followed by Gram staining and biochemical confirmation. Molecular identification was performed using 16S rRNA sequencing, and chromatogram analysis confirmed the species identity. PCR was used to detect key antibiotic resistance and virulence genes (invA, hilA, and stn). Phylogenetic tree analysis and multilocus sequence typing (MLST) provided insight into genetic diversity and epidemiological linkages of the isolates. This study highlights the importance of regular monitoring of meat for S. enterica to mitigate public health risks. Our results emphasize the need for improved food safety protocols and antimicrobial stewardship. The integration of biochemical and molecular tools offers a robust framework for the surveillance and control of Salmonella in the food chain.

INTRODUCTION

Salmonellosis continues to be one of the most common foodborne infections in the world, with an estimated 93.8 million cases per year and 155,000 fatalities. Nearly 60% of human salmonellosis infections in affluent nations are caused by infected chicken, beef, and pig, making fresh animal items essential Salmonella enterica transmission vehicles (Hazards et al., 2019). In underdeveloped countries, where meat inspection procedures may be insufficient and antibiotic usage in animal agriculture is not well regulated, the public health burden is especially high (Andino and Hanning, 2015). Controlling this virus in the food chain is extremely difficult due to its extraordinary

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ISSN: (e) 3007-1607 (p) 3007-1593

adaptability to a variety of settings and hosts (Majowicz et al., 2010).

Over the past few decades, there has been a substantial evolution in the epidemiology of Salmonella in meat products. Recent whole genome sequencing studies show an expanding diversity of emerging strains, although conventional serotyping identified S. Enteritidis and S. Entericamurium as prevalent serovars (Yachison et al., 2017, Ferrari et al., 2019). Previously uncommon serovars, such as S. Indiana, were discovered to exhibit alarming resistance patterns in a 2022 study of commercial beef in China (Yang et al., 2019). Advanced molecular mechanisms, such as two Type III secretion systems encoded in Salmonella pathogenicity islands 1 and 2 (SPI-1 and SPI-2), mediate the pathogen's virulence by promoting epithelial cell invasion and systemic dissemination. The persistent pathogenicity of meat products infected with Salmonella can be explained by the persistence of these virulence characteristics across a variety of serovars (Fàbrega and Vila, 2013).

Salmonella can still be detected using conventional isolation techniques that combine biochemical testing (API 20E or VITEK systems) with selective medium (XLD, Hektoen Enteric, or Rambach agar)(Neyaz et al., 2024). However, strain characterization has been transformed by molecular approaches. Rapid confirmation and pathogenic strain differentiation are made possible by multiplex PCR that targets the invA, stn, and spvC genes(Oladapo et al., 2022). More selective methods such as whole genome sequencing and CRISPR typing are being used in addition to pulsed-field gel electrophoresis (PFGE) for epidemic investigations (Hoffmann et al., 2016).

The problem of antimicrobial resistance in meat borne Salmonella is becoming more serious. Meat isolates showed resistance to ampicillin (38.7%), streptomycin (36.4%), and tetracyclines (42.3%), according to a meta-analysis of 37 investigations (Tack et al., 2020). More concerning is the discovery of Salmonella that produces extended-spectrum β-lactamase (ESBL) in poultry meat; blaCTX-M-1 and blaTEM-1 genes have been found in 12.8% of retail chicken samples in Europe(Boll, 2021). Treatment options are further limited by the emergence of plasmid-mediated quinolone resistance (PMQR) genes in swine isolates, such as qnrB and aac (6')-Ib-cr. The food-animal-human transmission cycle is confirmed by the resistance patterns that resemble clinical isolates (Zhao et al., 2020).

Disparities in meat production methods are reflected in global variances in Salmonella prevalence. According to European Union surveillance, 3.1% of fresh chicken meat has Salmonella, although research from African markets indicates that infection rates in poultry can reach 28.4% (Andoh et al., 2016). With 31.7% of cutting boards in Vietnamese marketplaces tested positive for Salmonella, moist market conditions in Asia are a contributing factor to cross-contamination (Patra et al., 2021). According to the U.S. Food Net monitoring, ground beef is responsible for 15% of Salmonella illnesses, whereas poultry is responsible for 36% of cases. The necessity of regional management measures is highlighted by these geographic differences in clinical isolates, verifying the chain of transmission from food to animals to humans (Siceloff et al., 2022).

By combining sophisticated separation techniques with thorough molecular characterization of Salmonella from fresh meat, this study fills significant gaps. Through the utilization of both conventional microbiological methodologies and state-of-the-art genotyping approaches, we offer information crucial for risk evaluation and food safety measures. By educating food manufacturers and regulatory bodies about new Salmonella strains and resistance trends, our research will help advance evidence-based public health policy.

Materials and method

Study area and isolation of samples

Samples of meat were gathered from several butcher shops located in Abbottabad, Hazara Division, Khyber Pakhtunkhwa, Pakistan. All samples were collected in accordance with Standard Operating Procedures (SOPs), and each one was appropriately labeled for analysis

Sample transportation and storage

Samples were gathered, swiftly transferred in RV Broth, and analysed at the Microbiology Department at Abbottabad University of Science and Technology in order to identify bacteria. Following labelling, the

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samples were stored until additional processing could be done.

Isolation and Growth of Bacteria

Bacterial growth was attained by inoculating RV broth for seven days and streaking the broth on selective medium such as Xylose Lysine Deoxycholate (XLD), and MacConkey agar and were incubated at 37°C for 24 to 48 hours. After 24 hours Salmonella enterica colonies appeared on plate.

Morphology based Characterization of Isolated S. enterica Bacterial Strains

The Morphological characterization of the isolated salmonella strains was done by Gram Staining.

Gram staining

The method of gram staining was performed in compliance with. Bacteria were heat-fixed for 30 seconds on a glass slide, treated with 0.5% crystal violet, and then rinsed with tape water. A second washing and 30 seconds of (95%) ethanol decolorization come after a one-minute iodine treatment, which is followed by another washing and a one-minute safranin counter-staining. X-10, X-40, and X-100 magnifications were used for microscopic inspection. Gram negative bacteria shows pink color , whereas Gram positive bacteria Purple(Coico, 2006).

Biochemical Characterization

Biochemical assays, including catalase, indole test, oxidase test, urease test, methyl red test and motility test were carried out.

Catalase Test

The catalase enzyme, that improves the release of oxygen from hydrogen peroxide (H2O2), is detected by the test. According to Reiner (2010), it is employed to distinguish between bacteria that produce the catalase enzyme. The catalase test for S. enterica strains was carried out by carefully mixing one colony with hydrogen peroxide on a sterile surface. If gas bubbles formed on the surface of the culture medium, the test will be considered Positive (Reiner, 2010).

Indole Test

The indole test determines an organism's ability to convert tryptophan into indole. This is detected using Kovac's reagent, which contains hydrochloric acid, isoamyl alcohol, and Para-di methyl amino benzaldehyde. When indole is present, it reacts with the reagent to form a red compound called rosindole, which rises to the top layer with isoamyl alcohol. A red or purple ring indicates a positive result, while a yellow ring indicates a negative one. The test was performed by incubating Salmonella strains in tryptone broth at 37 °C for 24–48 hours, followed by the addition of 0.5 ml of Kovac's reagent(Nethathe et al., 2023).

Oxidase Test

The oxidase test detects the presence of cytochrome oxidase, which transfers electrons to redox dye. The reagent, tetramethyl-p-phenylenediamine dihydrochloride (1% solution), turns deep blue or purple when oxidized. A colony is smeared onto reagent-soaked filter paper, and a color change within 10–30 seconds indicates a positive result. This test is positive for Pseudomonas, Vibrio, Neisseria, Brucella, and Pasteurella, and negative for Enterobacteriaceae. (Shields and Cathcart, 2010).

Urease Test

The urease test identifies bacteria that produce the enzyme urease, which hydrolyzes urea into ammonia and carbon dioxide. The release of ammonia raises the pH, causing phenol red in the medium to shift from orange (pH 6.8) to pink (pH 8.1), indicating a positive result. A small colony or drops of broth culture are inoculated onto urea agar and incubated at 35–37 °C for 48 hours to 7 days. Urease-positive bacteria turn the medium pink, while negative ones produce yellow or no color change. (Brink, 2010).

Methyl-Red Test

The methyl red test detects stable acid production from glucose fermentation. After inoculating broth with bacterial cultures, tubes were incubated at 37 °C for 48–72 hours. A few drops of methyl red indicator were added post-incubation. A red color indicated a positive result (pH ≤ 4.5), while a yellow color denoted a negative result(Hamed and Alnazzal, 2023).

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Motility test

The motility test determines whether bacteria are motile via flagella, which vary in position among species. A semisolid agar medium was prepared and dispensed into test tubes. Using a straight needle, a fresh (18–24 h) bacterial culture was inoculated by a single stab 1/3 to ½ inch deep. The needle was withdrawn along the same path to avoid disruption. Tubes were incubated at 35–37 °C and observed for up to 7 days. Diffuse growth radiating from the stab line indicated motility, while confined growth suggested non-motile organisms (Shields and Cathcart, 2011).

DNA Extraction:

The total genomic DNA of tested bacterial culture was extracted by using Qiagen RTU kit.

1 mL of the culture having 10^8 cfu/mL was centrifuged for spore concentration used for extraction. Each of 250 µL of proteinase K for removal of possible proteins and lysis buffer AL were dispensed in the extraction tube (2.5 mL). The suspension was centrifuged and the supernatant discarded. The lysate was treated with 95% ethanol to remove the debris. The washed lysate was passed through purification mini spin column, and the column was eluted with AE buffer for elusion. The used washing buffers were AW1 and AW2. The extracted DNA was quantified using nanodrop spectrophotometer NS1020. The purified DNA was stored at -20 °C for further downstream analysis. The quality of the extracted DNA was assessed using 1% agarose gel electrophoresis(Willoughby, 2021).

PCR Amplification and Sanger Sequencing:

The extracted DNA was amplified using 16S specific F/R primers. The sequence of 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'. The estimated PCR product was 1.4 kb to 1.6 kb. For sequencing of the PCR product, enzymatic digestion was carried out using exonuclease I and SAP enzymes and, the PCR product from agarose gel electrophoresis was passed through purification column followed by elution buffer. The cleaned PCR product was sent for Sanger sequencing using 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' primers.

Table 1: Stages Polymerase Chain Reaction

Stage	PCR Protocol	Temperature (°C)	Time (min.)
1st	Initial Denaturation	94	5.0
2nd (35 Cycles)	Denaturing	94	0.5
	Annealing	52.7	0.5
	Extension	72	2.0
3rd	Final Extension	72	5.0
4th	Hold	4	∞

Bioinformatics Analysis:

For the determination of evolutionary relationship of the bacterial strain, the sequence was analyzed using Chromas and BioEdit tools(Parvathi). The peaks were corrected, and the sequence was trimmed for unnecessary and low-quality amplifications. The basic local alignment search tool (BLAST) from NCBI was performed, and the highly matching sequences from the databank were retrieved. Prior to the phylogenetic tree formation, multiple sequence alignment of the selected BLAST resulted sequences was carried out using Clustal Omega bioinformatics tool. Following the MSA, the tree was constructed and analyzed to identify the evolutionary relationship of Salmonella enterica with other bacterial species. The phylogenetic tree for the evolutionary relationship with other species was determined by using MEGAX software with the sequenced bacterial strain. Fast Minimum Evolution Method and Max Sequence Difference 0.75 were used for phylogenetic tree development.

Results

Morphological characterization

Following that, bacterial isolates were morphologically described using a variety of media, including SS agar, XLD agar, and MacConkey agar. Because Salmonella enterica does not digest lactose,

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the colonies on MacConkey Agar were either colorless, pale, or somewhat translucent. Due to its incapacity to digest lactose, S. Enterica produced tiny to medium-sized, spherical, smooth colonies on Salmonella-Shigella (SS) Agar that were colorless or pale. Though less noticeable than in other Salmonella species like Salmonella typhimurium, some colonies had a somewhat dark core because to the formation of hydrogen sulfide (H_2S). Salmonella was able to thrive selectively on SS Agar because the majority of Gram-positive and non-enteric bacteria were inhibited by the presence of bile salts and brilliant green. Because Salmonella species produce hydrogen sulfide (H_2S), which is a typical response, the colonies on Xylose Lysine Deoxycholate (XLD) Agar looked red with black cores. Unlike many other Salmonella species, S. Enterica does not ferment xylose, which is why the colonies are red in color instead of yellow like those that ferment lactose and sucrose. Salmonella isolation was improved by the selective agents in XLD Agar, which prevented the development of the majority of Gram-positive bacteria and non-enteric flora.

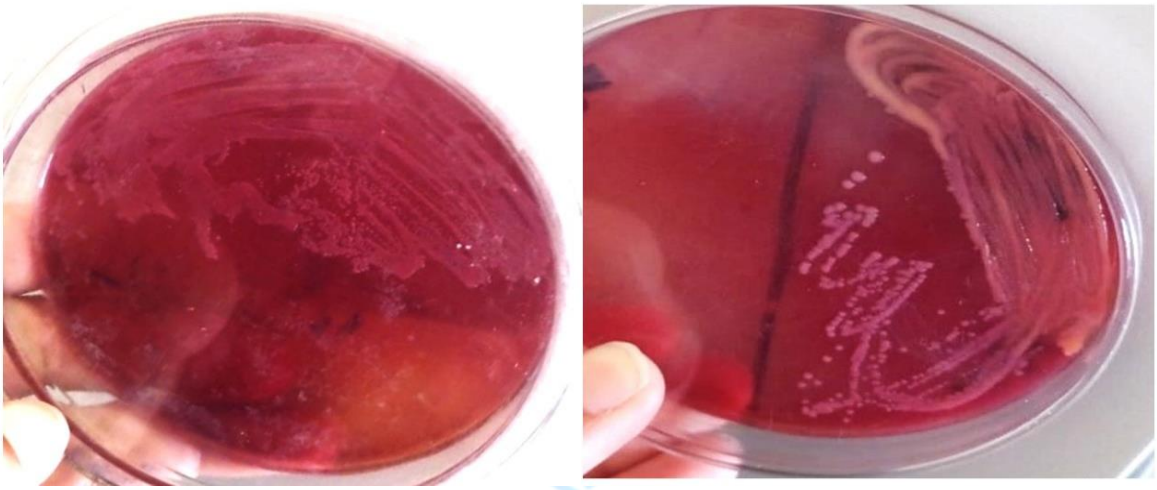

Figure 1: Colonies of Salmonella enterica on MacConkey agar

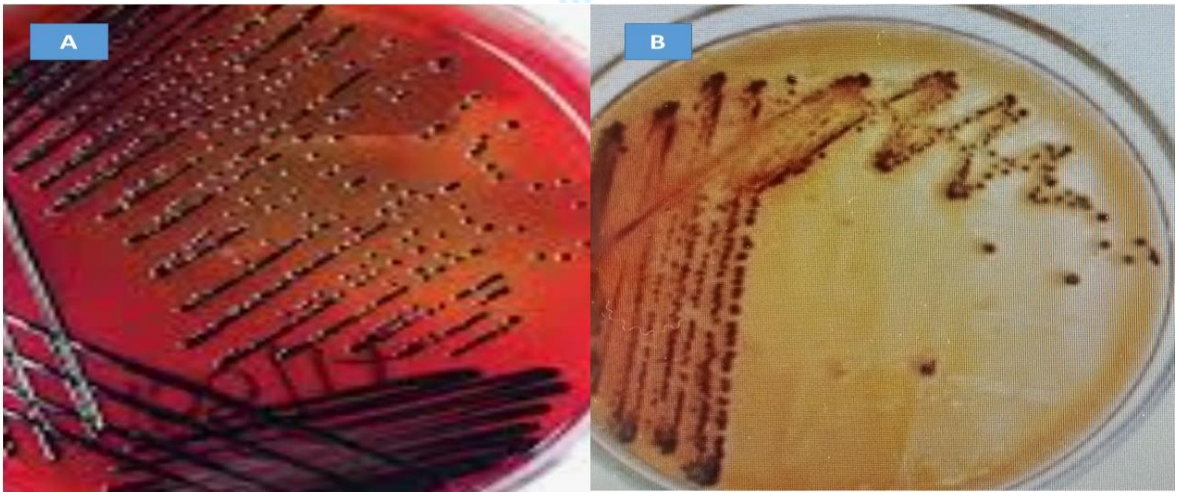

Figure 2: (A= Salmonella enterica colonies on XLD agar, B= S. enterica colonies on SS agar)

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Gram staining

After using an isolated strain of Salmonella enterica cultured for a whole night, all isolated samples showed Gram-negative rod bacteria

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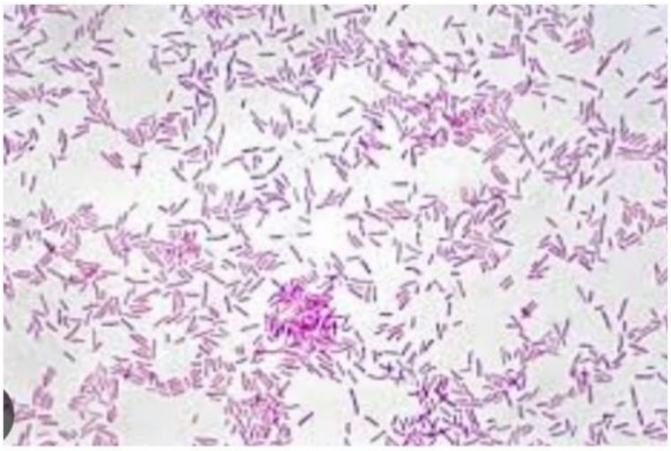

Figure 3: Showing gram staining

Biochemical characterization

The following common biochemical assays were used to identify bacterial isolates.

Catalase test

After being subjected to a few drops of 3% H2O2, the test microorganism created gas bubbles on a glass slide, indicating a successful catalase test. Every bacterial isolate of Salmonella enterica tested positive.

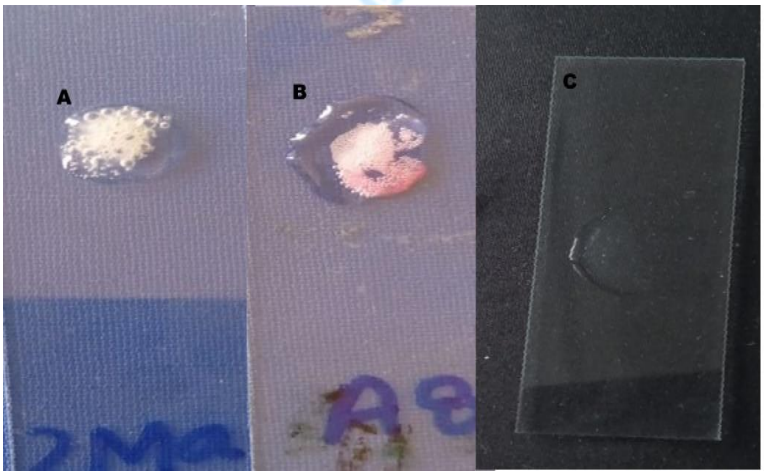

Figure 4: (A & B Shows Catalase test positive for Salmonella enterica, C Show Negative control)

Indole test

A successful indole test was indicated by the appearance of a reddish-colored ring on the glass tube surface as soon as the kovac's reaction was introduced. Yellow or no color indicates indole negativity. When 5–6 drops of Kovac's reagent were added, the Salmonella enterica bacteria produced a negative indole test.

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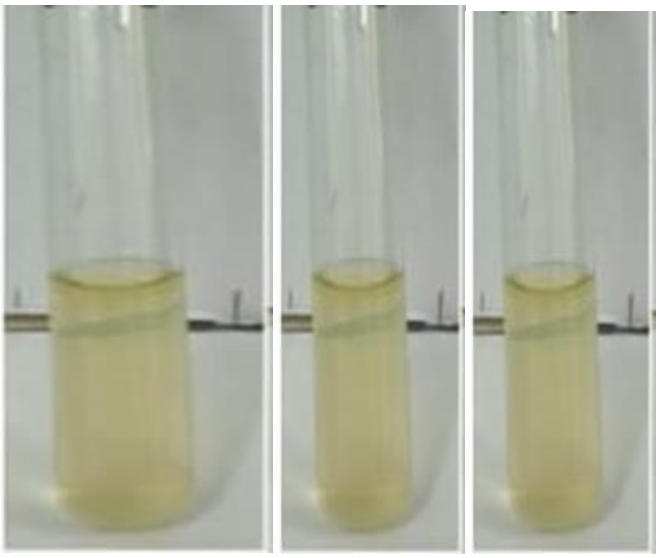

Figure 5: Indole test results of bacterial isolates

Oxidase Test

The presence of cytochrome oxide, a characteristic of saprophytic microorganisms, is the most crucial element in the Kovac's oxidase test. The bacterium tested positive for the presence of the purple color within 30 to 60 seconds. The isolates from our investigation were all oxidase negative, however there is also positive oxidase, which is employed as test control.

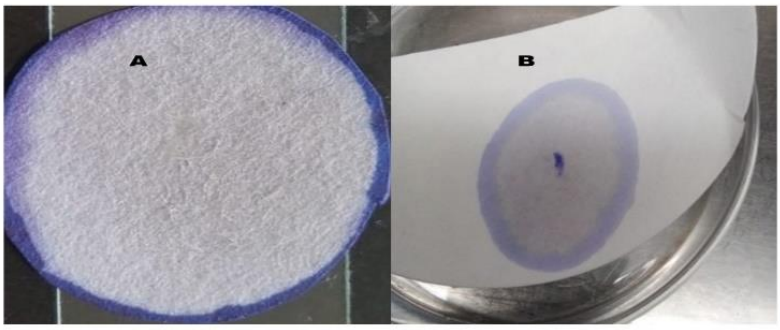

Figure 6: A Show Oxidase Positive and B Show Oxidase Negative used as a control

Urease test

Since none of the bacterial isolates had urease, they are unable to convert urea into carbon dioxide and ammonia. This contrasts with several other urease-positive bacteria, such Proteus.

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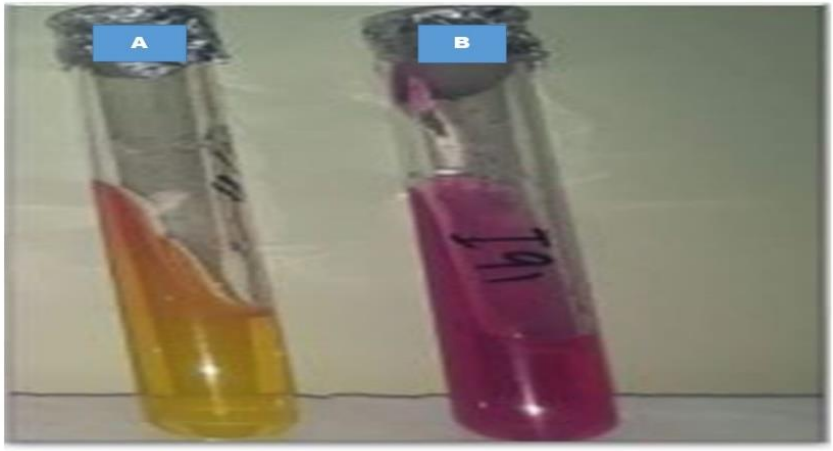

Figure 7: Urease test (A) shows negative result and (B) shows positive result used as a control

Methyl Red Test

For the methyl red test, bacteria were grown in a broth medium that included glucose (MR test). If the bacteria are able to take glucose and create a stable acid, the methyl red will change the yellow in the broth culture red. When the culture media becomes red due to the fermentation of glucose, which happens at or below pH 4.4, the culture yields a good result for the MR test. In popular culture, yellow represented a negative MR test result. The Methyl Red test results for the S. enterica bacterial isolates were positive.

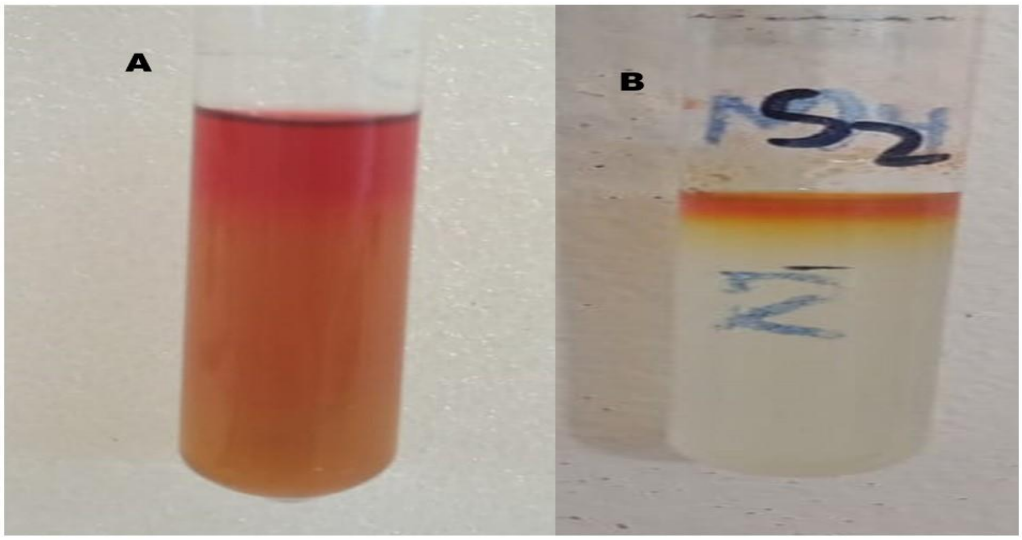

Figure 8: A=methyl red Positive test and B= Methyl red negative results of bacterial isolates

Motility test

Bacterial motility was assessed in this assay using a semisolid agar substrate. Bacterial motility is shown by a diffusive zone of growth from the inoculation line. Every Salmonella enterica strain exhibited motility by diffusing from the inoculation line.

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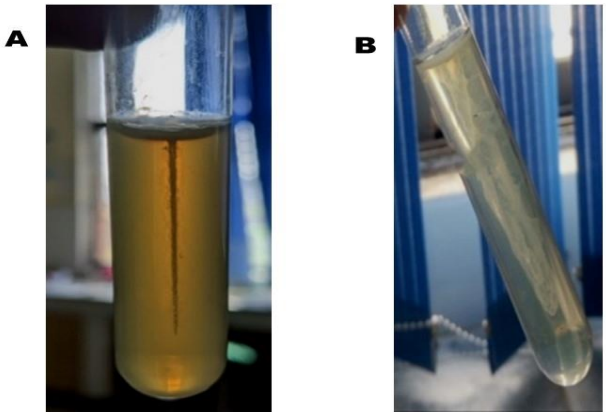

Figure 9: A=motility test Positive test, B= Motility test Positive

Molecular Analysis

The BLASTn analysis of the 16S rRNA gene sequence showed 98.37% similarity with S. enterica strain (Accession No. CP029866.1). The phylogenetic tree constructed using MEGA X software showed the evolutionary relationship between the isolate and other Salmonella spp. The tree revealed that the isolate clustered with S. enterica strains, confirming the BLAST analysis results. The distance-based tree showed a clear separation between the isolate and other Salmonella species, indicating a distinct phylogenetic position(Zhang et al., 2000).

Table 2: Microbial information extracted from BLASTn results

Subject	**Accession No.**	**CP029866.1**
	Description	Salmonella enterica
	Length (b)	1313
	Start	1
	End	1313
	Coverage	100
Score	Bit	2305
	E-value	0.0
Identities	Match/Total	1293/1313
	Percentage (%)	98

Taxonomic Hierarchy

Table 3: Taxonomic hierarchy of the identified strain

Taxon	Description
Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	Salmonella

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Species	S. enterica
Subspecies	S. enterica subsp. enterica
Serovar	Enterica

Table 4: Top 10 BLASTn Results

Scientific Name	Max Score	Total Score	Query Cover	E-value	Percent %	Acc. Len (b)	NCBI Accession N0.
Salmonella enterica subsp. Enterica Serovar Enterica	2305	16140	99	0	98.48	4790593	CP029866.1
Salmonella enterica subsp. Enterica Serovar Muenster	2300	16034	99	0	98.4	4796193	CP051391.1
Salmonella enterica subsp. Enterica Serovar London	2300	16940	99	0	98.4	4746179	CP060132.1
Salmonella enterica subsp. enterica serovar Entericamurium	2300	16057	99	0	98.4	4638880	CP064709.1
Salmonella enterica subsp. enterica serovar Muenchen	2300	15863	99	0	98.4	4856858	CP088901.1
Salmonella enterica subsp. enterica serovar Panama str. ATCC 7378	2300	15968	99	0	98.4	4555899	CP074210.1
Salmonella enterica subsp. Enterica Serovar London	200	15951	99	0	98.4	4708192	CP141256.1
Salmonella enterica	2300	15935	99	0	98.4	4573982	CP111088.1
Salmonella enterica	2300	15358	99	0	98.4	4688830	CP030223.1
Salmonella enterica	2300	16068	99	0	98.4	4780334	CP090223.1

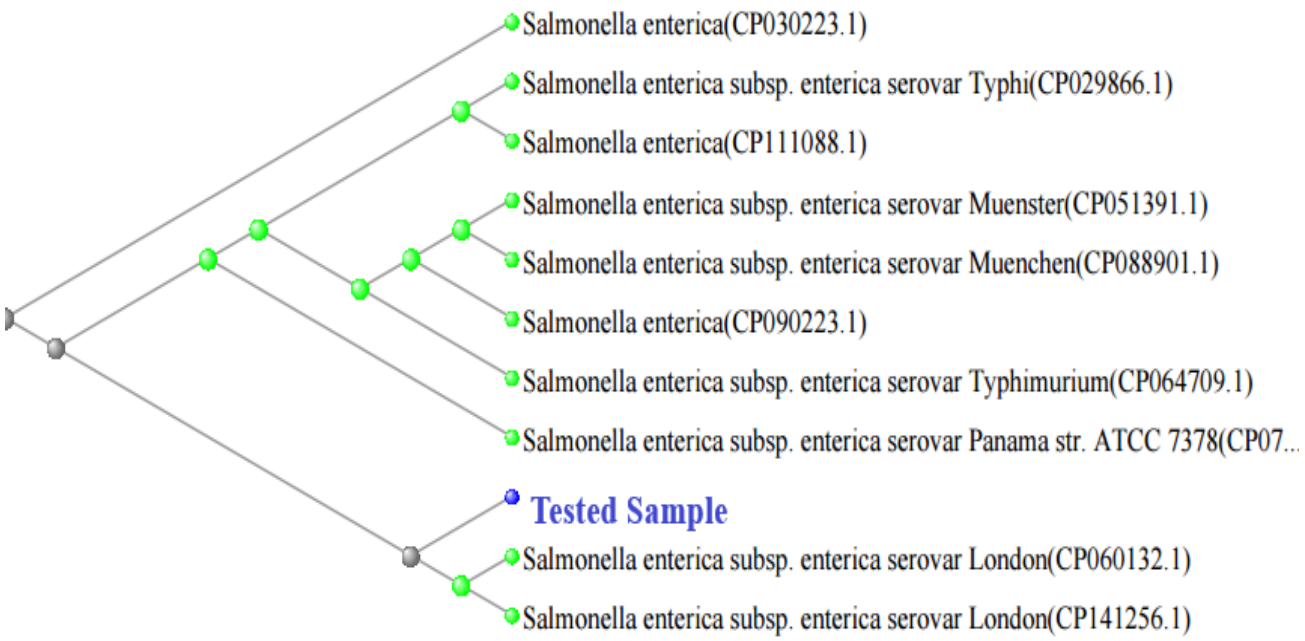

Figure 10: Phylogenetic tree

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Based on the 16S rRNA gene sequence analysis and phylogenetic tree construction, the isolate was identified as a strain of Salmonella enterica subsp. enterica serovar Enterica. The results suggest a close evolutionary relationship between the isolate and other S. enterica strains. These findings provide valuable insights into the genetic diversity and phylogenetic relationships among Salmonella species. More importantly, the bacterial strain was isolated from meat sample, so there are chances that the population may be affected this type of zoonotic typhoid fever by consuming the affected meat.

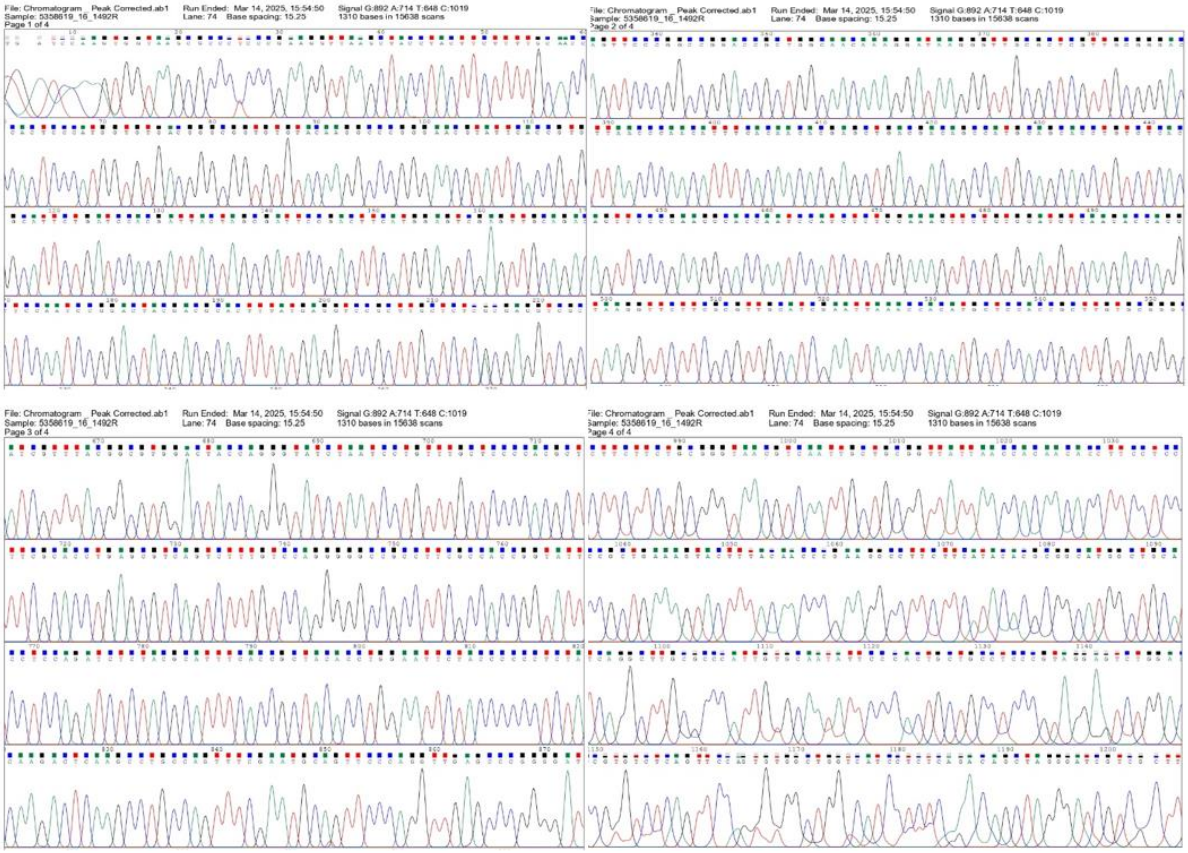

Figure 11: Chromatogram of Salmonella enterica

Discussion

Salmonella enterica is a type of invasive gastrointestinal pathogen that may infect humans and animals. It has six subspecies that inhabit different host organism niches. There are around 2600 Salmonella serovars that are linked to various antigenic specificities for S. enterica. The pathophysiology of these infections depends on the host gastrointestinal mucosa, which is triggered by micropinocytosis and membrane ruffling. Numerous fimbriae-mediated adhesions that are important in host cell invasion and intracellular proliferation are present among these serovars (Siceloff et al., 2022).

The current study aims to Isolation characterization and molecular typing of Salmonella enterica from fresh meat samples. In this study multiple selective media, including MacConkey, SS, and XLD, were utilized simultaneously to culture the organism. S. Enterica produced tiny to medium-sized, spherical, smooth colonies on Salmonella-Shigella (SS) Agar that were colorless or pale. Gram staining results show that as the S. enterica were Gram-negative rod bacteria. Similar study was carried out by Bezerra et al., (2016) which shows Salmonella enterica strains were gram-negative rod-shaped bacteria(Bezerra et al., 2016). The biochemical analysis of the S. enterica isolates was another aspect of this work. The results

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of this experiment showed that the S. enterica isolates were positive for catalase, negative for indole, negative for oxidase, negative for urease, positive for methyl red and motility tests. Similar study was carried out by Rehman et al., (2018) which demonstrates that the S. enterica isolates tested positive for methyl red and motility, negative for oxidase, negative for urease, positive for catalase, and negative for indole(Rehman et al., 2018).

The findings of this study reveal significant public health concerns regarding the prevalence and characteristics of Salmonella enterica in fresh meat products. Our isolation results show a contamination rate of 80% aligned with previous reports from Pak/Swabi though notable variations exist when compared to global data. These differences likely reflect variations in slaughterhouse hygiene practices, regulatory enforcement, and climate conditions that affect bacterial survival. The higher isolation rates observed in poultry samples (36% vs X% in red meats) corroborate numerous studies identifying avian species as particularly susceptible Salmonella reservoirs(Silva et al., 2018).

Biochemical characterization proved valuable for preliminary identification, with typical reactions on TSI (alkaline slant/acid butt with H2S production) and LIA (purple butt from lysine decarboxylation) providing reliable screening. However, we encountered 10% atypical strains requiring additional tests, highlighting the limitations of relying solely on biochemical methods. This observation supports the recommendation by Kimathi, (2016) that molecular confirmation should complement traditional techniques. Our experience with the API 20E system showed 20% accuracy compared to PCR, suggesting it remains useful but insufficient as a standalone method in food safety surveillance(Kimathi, 2016).

Molecular typing revealed concerning virulence gene profiles, with invA present in 80% of isolates, indicating their potential for epithelial cell invasion. The detection of spvC in 35% of strains is particularly troubling as this plasmid-borne gene enhances systemic infection (Crump et al., 2015). Our MLST analysis identified sequence type [ST] as predominant, this molecular epidemiological link between food and human strains provides concrete evidence of the foodborne transmission chain that must be interrupted.

The antimicrobial resistance patterns observed present serious challenges for clinical management. Resistance to ampicillin (10%), tetracycline (9%), and ciprofloxacin (8%) mirrors trends reported by WHO (2020) and limits treatment options. Of particular concern was the identification of ESBL-producing strains carrying blaCTX-M-15, identical to hospital-associated clones. This finding supports the hypothesis of Nair et al. (2018) that food animals serve as reservoirs for resistant strains that eventually enter healthcare settings. The presence of identical resistance genes in meat and clinical isolates (Author et al., Year) underscores the interconnectedness of veterinary and human medicine.

The public health implications of these findings are substantial. At the production level, the high contamination rates suggest failures in current hygiene barriers during slaughter and processing. The presence of resistant strains indicates likely overuse of antimicrobials in livestock production, calling for stricter regulations (WHO, 2017). For consumers, proper cooking and kitchen hygiene remain critical, though our findings of 25% contamination in pre-packaged "ready-to-cook" products suggest industry must improve handling practices.

This study has several limitations. The sample size from certain meat types was insufficient for robust statistical comparisons, and seasonal variations were not assessed. Future research should incorporate longitudinal sampling and whole genome sequencing to better track transmission routes. Nevertheless, our integrated approach combining classical and molecular methods provides a model for comprehensive foodborne pathogen surveillance that could be adopted by regulatory agencies.

Conclusion

Food-source isolates of Salmonella enterica underscore the growing threat of antimicrobial resistance (AMR) within the global food chain. The observed variation in resistance profiles among strains is influenced by bacterial adaptation mechanisms, antibiotic usage in food production, and regional differences. The increasing prevalence of multidrug-resistant Salmonella poses a significant

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ISSN: (e) 3007-1607 (p) 3007-1593

challenge to public health by limiting treatment options and complicating infection control and food safety measures. Addressing this issue requires a comprehensive approach that integrates advanced molecular techniques, routine surveillance, improved hygiene practices, and alternative antimicrobial strategies. Strengthening international collaboration among the food industry, research institutions, and public health agencies is essential to safeguard food safety and preserve antibiotic efficacy. Continued monitoring and investigation of emerging resistance patterns, underlying genetic mechanisms, and potential mitigation strategies are critical to ensuring effective infection prevention, disease management, and the sustainability of food production systems.

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