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Research Article

Evaluation and Sequencing of 16S rRNA Gene for Molecular Identification of Prevalent *Salmonella* Strains Isolated from Food and Water

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Abstract

Salmonella is a leading cause of intestinal illnesses globally and is also responsible for severe systemic diseases such as typhoid and paratyphoid fevers, often transmitted through contaminated food. Understanding the prevalence and genetic diversity of *Salmonella* is essential to addressing its public health impact. This study investigates the prevalence and genetic diversity of *Salmonella* strains in food and water samples from Karachi, with a focus on the 16S rRNA gene for phylogenetic analysis and the *InvA* gene as a virulence marker. A total of 110 food and water samples were analyzed using microbiological assays, PCR, gene sequencing, and phylogenetic grouping. The 16S rRNA gene, known for its evolutionary stability, was employed to identify bacterial species and trace phylogenetic relationships. The *InvA* gene, a key virulence factor, was detected to assess pathogenic potential. Gene sequencing and phylogenetic analysis were conducted using MEGA-X software to construct a Maximum Likelihood phylogenetic tree incorporating newly sequenced and reference strains. The study identified *Salmonella* isolates and revealed their evolutionary relationships and geographic origins. The phylogenetic analysis demonstrated genetic diversity among the isolates, highlighting their potential public health implications. The findings provide valuable insights into the genetic diversity of *Salmonella* in food and water samples from Karachi. These results underline the importance of molecular surveillance in understanding *Salmonella* evolution and controlling its public health impact.

Keywords: *Salmonella*, genetic diversity, phylogenetic analysis, 16S rRNA, *InvA* gene, public health.

1. Introduction

Infections caused by *Salmonella* are considered a major health concern, accountable for creating substantial illness and economic loss globally. Every year millions of *Salmonella* infection cases are reported worldwide (Miladi et al. 2016). The genus *Salmonella* mainly comprises two species, *Salmonella* enterica and *Salmonella* bongori. Among these *S.* enterica is responsible for causing life-threatening infections such as gastroenteritis, endocarditis, septicemia, and meningitis. *Salmonella* enterica is categorized into six species which contain more than 2600 serotypes (serovars) (Zhu et al. 2014). Typhoid fever, a systemic infection caused by *S.* enterica serovars Paratyphi A, Paratyphi *B*, and Paratyphi

C as well as *S.* enterica serovar Typhi, has been a serious health concern for decades (R. Johnson, Mylona, and Frankel 2018). According to a recent study on the global impact of typhoid fever, between 200,000 and 600,000 deaths and 26.9 million illnesses are reported annually (Qamar, Ismail, and Akhtar 2020). A comprehensive study from four developed countries revealed that the prevalence of diarrheal disorders increased from 0.44 to 0.99 cases per person-year (Donkor 2013; JUNCU et al. 2021). The World Health Organization (WHO) has created the Foodborne Disease Burden Epidemiology Reference Group to provide global indicators of foodborne disease (Parisi et al. 2020; Donoso, Paredes, and Retamal 2020). However, developing countries, including Pakistan, bear the highest burden of such infections. Pakistan has the second-highest annual incidence of typhoid fever, with 412.9 cases (per 100,000 person-years) annually (Gwaza and Adie 2024). The WHO estimates Pakistan faces the highest global burden of typhoid fever, with 11 million cases and more than 60,000 deaths annually (Parveen and Khan 2023). While several virulence factors contributing to the pathogenesis of *Salmonella* have been identified, the molecular diversity of locally circulating strains in regions such as Karachi remains underexplored. Despite advancements in detection and molecular characterization techniques, there is limited data on the prevalence, genetic diversity, and phylogenetic relationships of *Salmonella* strains isolated specifically from Karachi. Most studies have not fully utilized modern molecular tools, such as the 16S rRNA gene as a universal molecular marker and virulence-specific markers like the *InvA* gene, for the identification and phylogenetic analysis of local *Salmonella* isolates. Addressing this gap which is our primary objective, could provide critical insights into the evolutionary patterns, geographic origins, and public health implications of these strains, thereby aiding in the development of targeted prevention and control strategies.

2. Materials and Methods

2.1. Sample Collection and Initial Processing

A total of 110 *Salmonella* strains were collected from food and water samples across various locations in Karachi. The samples were pre-enriched to enhance bacterial recovery by inoculating them into Tryptic Soy Broth (TSB) (OXOID) and incubating them overnight at 37°C (ul Huda et al. 2024).

2.1 Selective Isolation and Identification

After overnight pre-enrichment, a loopful of culture was aseptically streaked onto Xylose Lysine Deoxycholate (XLD) (OXOID) agar, a selective and differential medium designed for the detection and preliminary identification of *Salmonella*. Plates were incubated at 37°C for 24–48 hours. Colonies exhibiting characteristic *Salmonella* morphology on XLD agar (red colonies with black centers) were considered presumptive positive. For samples that did not exhibit sufficient growth on XLD agar, additional selective and differential media were utilized, including Hektoen Enteric (HE) agar (OXOID) and Bismuth Sulfite Agar (BSA). On HE agar, *Salmonella* typically produces greenish-blue colonies with black centers, while on BSA, they appear as black, shiny colonies due to hydrogen sulfide (H2S) production (ul Huda et al. 2024).

2.2 Biochemical Confirmation

To confirm the identity of the isolates, presumptive colonies from the selective media were subjected to biochemical testing. Each isolate was inoculated onto Triple Sugar Iron (TSI) agar slants and incubated at 37°C for 18–24 hours. The TSI test provides critical diagnostic information based on the organism's ability to ferment glucose, lactose, and sucrose, and produce gas or hydrogen sulfide. *Salmonella* isolates typically exhibit an alkaline slant (red) and acidic butt (yellow) with H2S production, resulting in blackening of the medium (ul Huda et al. 2024).

2.3 Storage of Isolates

Confirmed *Salmonella* isolates were suspended in glycerol (30%) and stored at -20°C to maintain

viability for downstream molecular and phylogenetic analysis.

2.4 Extraction of DNA from *Salmonella*

An isolated colony from XLD agar was selected, picked, and then immersed in 100◎l DNase-free water in an Eppendorf (R. L. Johnson et al. 2021). The DNA from the cell was then extracted by submerging the Eppendorf in an 80°C water bath for minutes followed by centrifugation and collection of supernatants in separate DNase-free Eppendorf (Khan et al. 2014). This suspension was then placed in a freezer at -4°C and used as a DNA template of *Salmonella* for colony PCR reaction that would be conducted for two particular genes (R. Johnson, Mylona, and Frankel 2018).

2.5 Detection of 16S rRNA Gene

The 16S rRNA gene, a conserved region in bacteria, is commonly used as a molecular marker for bacterial identification. In this study, universal primer pairs 27F and 1492R were used to amplify an approximately 1350 bp fragment of the gene (Table 1). These primers are well-established for bacterial detection and identification (Ramanathan Srinivasan et al. 2021). The PCR reaction was performed by preparing a total volume of 25 µl in a PCR tube, which included 1 µl of forward primer (27F) (ThermoFisher), 1 µl of reverse primer (1492R), 4 µl of extracted DNA sample, 19 µl of DNase-free water, and 25 µl of master mix. The reaction was carried out at low temperatures and maintained using an ice tray. PCR amplification was performed using a thermal cycler with 35 cycles, beginning with an initial denaturation at 95°C for 5 minutes, followed by denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, initial extension at 72°C for 90 seconds, and a final extension at 72°C for 10 minutes. The amplified PCR products were visualized on a 2% agarose gel under UV light, confirming the successful detection of the 16S rRNA gene (Ramya Srinivasan et al. 2015; Nomura et al. 2023).

2.6 Detection of *Inv-A* (Invasive A) Gene

For the amplification of the *InvA* gene, specific primers, S139-F and S141-R, were used, producing an amplified fragment of approximately 284 bp (Table 1) (Allam et al. 2019). The PCR setup was conducted under low-temperature conditions, similar to previous protocols, by placing an ice tray beneath the PCR tube rack. The reaction mixture included 1 µl of forward primer, 1 µl of reverse primer, 12 µl of master mix, 8 µl of DNase-free water, and 3 µl of DNA sample, making a total reaction volume of 25 µl. The PCR tubes were placed in a thermocycler programmed as follows: an initial denaturation at 94°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 7 minutes (Anejo-Okopi et al. 2016; Allam et al. 2019). The amplified PCR products were visualized through gel electrophoresis. A 100 bp DNA ladder (Generuler) was loaded in the central well to aid in size determination. The gel was run at a voltage of 58 volts, ensuring clear separation of the amplified products.

2.7 16S rRNA Gene Sequencing

For this assay, 10 *Salmonella* isolates were selected for 16S rRNA sequencing, based on the results of *InvA* gene amplification. Isolates identified as possessing the pathogenic *InvA* gene were subjected to Sanger sequencing. Sequencing targeted a ~1350 bp fragment of the 16S rRNA gene, which had been previously amplified using colony PCR. The sequencing process was conducted by *Macrogen*, and the resulting sequences were subsequently aligned for further analysis (Timme et al. 2013).

2.8 Sequence Alignment by MEGA X

The obtained sequences were then aligned by using MegaX (Molecular evolutionary genetics analysis software (Winand et al. 2019). Downloaded sequences were confirmed as

Table 1: Primer sequences used for *Salmonella* detection.

PRIMER	TARGET REGION	SEQUENCE 5′-3′	SENSE	AMPLICON SIZE
16SrRNA	27F	AGAGTTTGATCCTGGCTCAG	+	250bp
	1492R	GGTTACCTTGTTACGACTT	_	
Inv-A				
	S139-F	GTGAA ATT ATC GCC ACG TTC GGG CAA	+	284bp
	S141-R	TCAT CGC ACC GTC AAA GGA ACC	_	

Salmonella by following steps; the sequences were matched by adding 27F and 1492R primers used for 16S rRNA. Then BLASTn was performed to check if the sequences really match other existing strains of *Salmonella*. After the BLASTn was performed, the obtained sequences were trimmed keeping the most similar strains in consideration (to provide the highest percentage of query that will further aid during submission for accession number to NCBI). All of this aligned/trimmed data was simultaneously saved as a particular session in MEGAX. Another software that was used to check whether the chimeric sequences if are present or not, is DECIPHER 2.20.0 (Chimeras are two or more biological sequences that are overlapping or incorrectly joined together) (Wang et al. 2007). After checking the obtained sequences for similarity and chimeric sequences, a list was made for the closely related strains of *Salmonella* to generate a phylogenetic tree.

2.9 Matching & Accession Number

For accession number, online submission was made to NCBI by including the sequences of the isolated that were sequenced, matched, and precise after the number of steps described above.

2.10 Bootstrap Method

To create a phylogenetic tree, there are a few ajor steps. Identification of the sequences that are found homologous to the sequence of interest. It was keenly decided which sequences are significant enough to include in the tree. Then an electronic file of the concerned sequences was downloaded. FASTA file among all was used in MEGAX. Sequence alignment that included precision, BLASTn, and matching was done. Then this resultant alignment was used to create a phylogenetic tree by Bootstrap method of maximum similarity.

3. Result and Discussion

3.1. Initial Screening of *Salmonella*

On initial screening, 24 samples out of 110 showed positive for *Salmonella* by convention method. On XLD agar, black-centered characteristic colonies were observed. On BSA typical brown-black colonies appeared and on HE agar blue-green colonies were seen. *Salmonella* isolates were confirmed through biochemical testing by streaking and stabbing on Triple Sugar Iron agar (TSI). All 24 samples were positive on TSI; Acidic slant, alkaline butt with H_2S production, and few of the samples showed positive gas production.

3.2. Confirmation of *Salmonella* by PCR

PCR was carried out for the 16S rRNA gene, followed by gel electrophoresis, which showed that 18 isolates gave a positive result for the 16S rRNA gene, as visualized by sharp bands under a UV trans-illuminator (Figure 1). It has been observed that phylogenetic analysis of some particular fragments of the 16S rRNA gene shows very notable hierarchic relations between genus

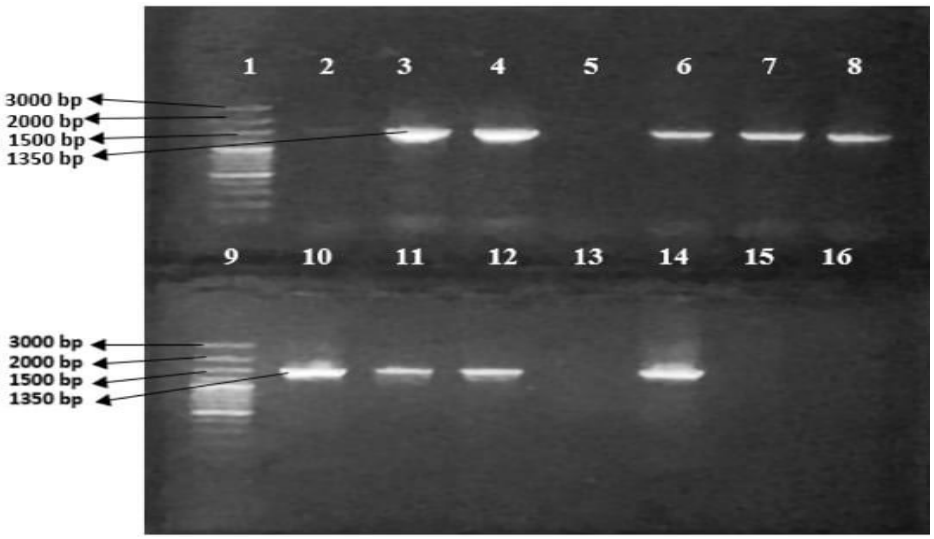

Figure 1: Gel electrophoresis image of colony PCR for detection of 16S rRNA using universal primer 27F and 1492R Lane 1 and 9 is Gene Ruler 100 bp plus DNA ladder (0.5 g/lane); Lane 2-8 and 10-16 depicting PCR results of 14 samples. Lane 3,6,10 and 11 are showing no amplified product whereas others are positive with 1350 bp amplified product.

and species of *Salmonella* and also has a key role in bacterial taxonomy and phylogenetic studies (Janda and Abbott 2007). To find genetic relatedness between the species, conserved portions of specific genes are always targeted to rule out the possibility of mutation. 16S rRNA gene is one of the frequent housekeeping genetic markers used in molecular identification. The significance of this gene lies in the fact that its function has not changed over time which suggests the random sequence changes are a more accurate measure of evolution in bacteria. The next step was the amplification of *Inv-A* using its specific primer. Out of all the *Salmonella* isolates, 10 tested positive for *Inv-A* after performing colony PCR. The number of positive isolates remained the same after repeating the specific PCR reaction to rule out any potential errors (Figure 2). The *InvA* gene is a widely recognized molecular target for the identification of *Salmonella* species. It serves as a critical marker for nucleic acid-based detection methods. The use of PCR targeting the *InvA* gene is particularly significant and is recommended as a reliable *Salmonella*-specific detection method in the Bacteriological Analytical Manual by the U.S. Food and Drug Administration (FDA) (Buehler et al. 2021). Approximately 93% of *Salmonella* strains responsible for human infections possess this virulence gene (*InvA*) (Amini et al. 2010). The *InvA* gene is chromosomally located and encodes an essential inner membrane protein that facilitates the invasion of host epithelial cells. This gene is highly conserved across most *Salmonella* serotypes, making it a critical target for detection and identification.

3.3. 16SrRNA Sequencing

A total of 10 isolates were selected for sequencing using the Sanger method. Based on Sanger sequencing with the universal forward primer 27F for the 16S rRNA gene, the sequences were matched to confirm the identity of the sequenced product. Partial (16S ribosomal RNA) gene sequences of *Salmonella* species were retrieved from NCBI, representing different geographical locations worldwide. Not all of the sequences

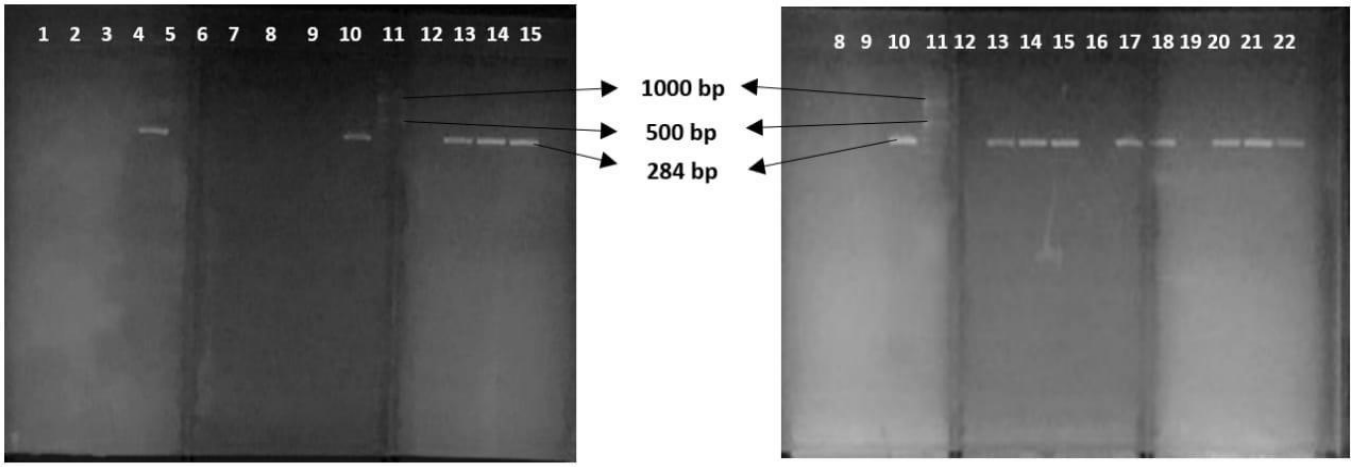

Figure 2: A representative gel electrophoresis image of Colony PCR for detection of *Inv-A* gene using primer S139- F and S141-R. Lane 11 is GeneRuler 100 bp plus DNA ladder; Lane 1 to 10 and 12-22 depicting PCR results of 21 sample. Lane 4, 10, 13, 14, 15, 17, 18, 20, 21 and 22 are showing positive results of amplification of a 284bp fragment whereas others are negative for amplification. Left and right depict the same gel that has been placed on the Trans illuminator with two angles to get all the bands. Large Gel having 22 wells was prepared).

matched *Salmonella*, despite the sequenced isolates being selected based on the presence of the *InvA* gene in their genomes. The *InvA* gene is a virulence factor that contributes to the pathogenicity of the bacterium. It encodes an invasion protein present in the bacterial cell membrane, enabling the bacterium to invade host epithelial cells and spread infection. This gene is part of pathogenicity island I, also known as *Salmonella* pathogenicity island I (SPI-1), and is actively present in all *S.* enterica serovars (Lyu et al. 2021). The discrepancy, where some sequenced isolates did not match *Salmonella*, might be attributed to the sequencing being conducted with a single primer. A more comprehensive hierarchical pattern of the organism could be achieved by sequencing with both forward and reverse primers (Pilar et al. 2020). After performing BLASTn for all 10 isolates, results showed significant diversity at the species level. Results from the standard BLASTn database showed that most sequences were identified as *S.* enterica, with high similarity to partial 16S rRNA sequences. For example, strains such as (acc. OM311260), 15, 16, 18, 22 (acc. OM171243), and 22mx exhibited more than 90% similarity to known *Salmonella* sequences (Figure 1). The BLASTn results for the first isolate, J01_13, revealed 93.03% similarity to *S.* enterica subspecies arizonae strain: GTC 1732, with approximately 200 bp of matching nucleotides. This sequencing can reveal phylogenetic features and assign gene functions based on sequence homology (Shariat et al. 2021). A detailed study of the phylogenetic tree can offer insights into how closely related the sequenced isolates are to those with known functions, either through biochemical or biological findings. In this study, a Bootstrap Maximum Likelihood (ML) phylogenetic tree was constructed based on the sequenced isolates (Figure 3). The ML approach starts with constructing an initial tree using a suboptimal but fast method, such as Neighbor-Joining (NJ). Branch lengths are then optimized for the given data and tree topology under the selected evolutionary model. Variations in topology are

generated using the Nearest-Neighbor Interchange (NNI) method to find better fits to the data. This iterative process continues until no further improvements can be made. The Tamura-Nei model and the maximum likelihood approach were used to infer evolutionary history. The evolutionary history of the studied taxa is represented by a bootstrap consensus tree, generated from 1,000 replicates. Branches supported by less than 50% of bootstrap replicates were collapsed. The proportion of duplicate trees where related taxa clustered together during the bootstrap test is shown next to the branches. For the heuristic search, pairwise distances were calculated using the Tamura-Nei model. The Neighbor-Joining and BioNJ algorithms were applied to create a starter tree, and the topology with the best log-likelihood value was selected. A total of 10 nucleotide sequences were analyzed, and the final dataset contained six sequences along with reference sequences from GenBank (NCBI). These sequences were used to construct the ML Bootstrap consensus tree using MEGA X. The sequences were also submitted to GenBank, and accession numbers (OM171243 and OM311260) were obtained. Bootstrap values in phylogenetic trees indicate the robustness of the branches. For example, a bootstrap value of 100% (out of 1,000 replicates) suggests high confidence that the branch is not due to random data points, whereas values around 50% indicate lower reliability. This study revealed the maximum hierarchy of the strains as much as possible with single-primer sequencing. However, phylogenetic identification can be improved by sequencing with both forward and reverse primers. *Salmonella* is a significant pathogen due to its high morbidity rate in the Asian region. The phylogenetic patterns of *Salmonella* strains isolated in Pakistan require further research to better understand their genetic relationships and evolutionary hierarchy. A thorough investigation into the genetic relatedness of these strains can help identify their position in the global context of this pathogen, which continues to pose a substantial public health burden.

Beyond molecular significance, the presence of *Salmonella* in consumable sources highlights the compromised quality of food and water, contributing to a high risk to public health. Ongoing research aims to better control the prevalence of *Salmonella* infections in Pakistan. Considering the high morbidity and mortality rates associated with this pathogen in Asia, *Salmonella* is recognized as an opportunistic pathogen that requires immediate and thorough investigation.

4. Conclusion

This study has revealed the species of *Salmonella* from diversified origins that are isolated from food and water samples in Karachi. A significant pattern highlighted in this research is despite all 10 isolates being positive for *Inv-A gene* (*Salmonella* pathogenicity Island gene present in 90% of strains), 4 isolates came out to not match *Salmonella* spp. in the NCBI database. This molecular significance apart, the presence of *Salmonella* in these consumable sources indicates the quality of food and water available to people is compromised contributing to a high risk to public health. Further research within the ambit of this field is still underway to control the prevalence of *Salmonella* infections in Pakistan.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Approval

The study was approved by the Institutional Review Board.

Consent Forms

Not applicable

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Data Availability

All the data related to this study is available with the authors.

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Authors Contribution

UJ, SU, RS, Zam, NH, and FZ carried out all the bench work, initial draft, and final manuscript. ZAM, MNK, and TS helped in data collection and statistical analysis. ZAM conceptualized and supervised the study.

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