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Antibiogram and Molecular Characterization of *Salmonella* species from Borehole Water in some Sub-Urban Communities of Rivers State, Nigeria

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ABSTRACT

Borehole water has remained a major source of domestic water in most urban and semi-urban settlements, and have been frequently associated with cases of Salmonella contamination. This study investigated the prevalence, antibiotic resistance profiles, and molecular features of Salmonella species recovered from borehole water sources in selected suburban communities of Rivers State, Nigeria. A total of 120 borehole water samples were randomly obtained from Elelenwo, Rumuokparali, and Eliozu communities within Obio/Akpor Local Government Area during four sampling periods representing different seasons of the year. Samples were analyzed using established microbiological procedures, and isolates were identified through cultural and molecular techniques. Antimicrobial susceptibility testing was carried out using the Kirby–Bauer disc diffusion method in accordance with CLSI standards to determine resistance patterns. Polymerase chain reaction (PCR) analysis was further employed to detect the presence of the antibiotic resistance gene blaTEM and the toxigenic gene stn among the isolates.The Salmonella isolates resistance showed 100% resistance Cefotaxime, Nitrofurantoin, Cefexime and Ceftriazone, with overall MAR index ranging from 0.25 to 1.0. The 16S rRNA analysis identified the relatedness of Salmonella enterica subspecies to montevideo strain 11TTUC-046, paratyphi C strain SA49, typhi strain PU4; typhi strain MSAR18, and enteritidis strain SA26. The study also revealed the presence of antibiotics resistance (blaTEM) and toxigenic (stn) gene markers in all the isolates. The study has shown the prevalence of different strains of Salmonella species with virulence gene biomarkers and their potentials to resist conventional antibiotics. The findings from this study therefore highlights the need for water treatment practices and regulated antibiotics stewardship.

Keywords: *Antibiogram; borehole water; Molecular characterization, Salmonella, Sub-Urban Communities.*

1. INTRODUCTION

Salmonella species are Gram-negative, rod-shaped, motile bacteria belonging to the family *Enterobacteriaceae* [1]. Numerous serotypes have been identified, many of which are capable of causing disease in humans [2]. According to the Kauffmann–White classification system, the genus *Salmonella* is grouped into two main species, *Salmonella enterica* and *Salmonella bongori* [3,4]. Among these, *S. enterica* is the species most frequently associated with human infections and is further divided into more than 2,600 serovars. The human-adapted serovars *S. Typhi* and *S. Paratyphi A* are responsible for enteric fever [5], whereas serovars such as *S. Typhimurium* and *S. Enteritidis* are common causes of non-typhoidal salmonellosis worldwide [6]. *S. enterica* is subdivided into six subspecies designated enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV), and indica (VI). The Roman numerals I–VI are used to denote these subspecies, while subspecies V is omitted because it previously referred to *S. bongori*, which has since been recognized as a separate species within the genus [7,8].

Conventional culture-based approaches for detecting *Salmonella* in water typically involve several sequential steps, including pre-enrichment, selective enrichment, and isolation on selective media such as *Salmonella–Shigella* agar, followed by biochemical characterization of suspected isolates. Although these procedures remain the standard in many laboratories, they are often labor-intensive, time-consuming, and occasionally yield inconsistent results [9]. To overcome these limitations, molecular diagnostic methods such as the polymerase chain reaction (PCR) have emerged as valuable alternatives or complementary tools for *Salmonella* detection. PCR assays have demonstrated superior sensitivity and specificity for identifying *Salmonella* in a variety of sample types, including blood, stool, and environmental materials [10]. The technique functions by amplifying a specific DNA region, allowing even minute quantities of genetic material to be detected and analyzed. During PCR, synthetic DNA primers are used to target and replicate a chosen segment of the bacterial genome. Through repeated cycles of heating and cooling, these fragments undergo exponential amplification, producing sufficient DNA for reliable analysis [11]. In addition to its precision, PCR offers faster turnaround times and greater reproducibility compared to traditional culture-based techniques, making it an indispensable tool in modern microbiological diagnostics.

Several factors have been recognized as drivers of increasing antibiotic resistance among strains. One of the methods of antimicrobial resistance in *Salmonella* species is the production of the *blaTEM* gene which plays a critical role in presenting resistance to *β*-lactam drugs [12] and this has played a great role in the failure of *Salmonella typhi* treatment in many countries. The most prevalent mechanism of resistance to β-lactam antibiotics involves the production of β-lactamases, enzymes that inactivate β-lactam compounds by hydrolyzing their characteristic four-membered β-lactam ring [13,14].

The *stn* gene, which encodes a heat-labile enterotoxin located on the chromosome of *Salmonella*, has been identified as an important virulence determinant contributing to the clinical symptoms observed in gastroenteritis and typhoid fever. [15]. Multiple studies have linked the stn gene to diarrhoea caused by *Salmonella* infection [16, 17]. The stn gene is recognized as a virulence factor in *Salmonella* and has been proposed as a potential contributor to diarrhoeal disease, as earlier studies demonstrate that it exhibits enterotoxic activity. Furthermore, studies have shown that a specific portion of the Stn protein (residues 127–142) exhibits structural resemblance to the active sites of cholera toxin (CT) and heat-labile enterotoxin (LT) ADP-ribosyltransferases. Based on this similarity, Stn is proposed to contribute substantially to acute gastroenteritis and diarrhoeal manifestations, playing a role in *Salmonella* virulence and its enterotoxic effects [18]. The study aimed at assessing the antibiogram and molecular characterization of *Salmonella* species in borehole water in some sub-urban communities of Rivers State, Nigeria.

2. Materials and Methods

2.1. Description of Study Location

The present study was conducted in Obio/Akpor Local Government Area (LGA) of Rivers State, Nigeria, which is among the 23 administrative LGAs in the state. The study locations were Rumuokparali (4.8637° N, 6.9190° E), Eliozu (4.8599° N, 7.0217° E), and Elelenwo (4.8398° N, 7.0727° E) communities in Obio/Akpor Local Government Area, Rivers State, Nigeria. Geographically, it is located at approximately 4.8776° North and 7.0283° East, The LGA spans an area of approximately 260 km^2. According to the 2006 census, the LGA had a population of 462,789. Its postal code is 500102, and it is constituted mainly by the people of the Ikwerre ethnic nationality.

2.2. Sample Size Determination

The study's sample size was calculated using the following formula [19]:

$N = [Z2(pq)]/d^2$

Where: N= required sample size

Z= Normal standard distribution that corresponds to confidence interval as 1.96

p= Prevalence of *Salmonella* species

q = 1-p d= expected level of precision at 0.05

2.3. Sample Collection

A total of 120 borehole water samples were collected randomly from three communities within Obio/Akpor Local Government Area, Rivers State. Ten samples were collected from each community during every quarter, resulting in 40 samples per community.

2.4. Transportation and preservation of samples

The collected samples were transported in an icebox maintained at 4ºC and were analyzed in the Microbiology Laboratory within six hours of collection, on the same day of sampling.

2.5. Bacteriological examination

One (1ml) of the sample was pre-enriched into 9mls of selenite broth and incubated at 37°C for 24 hours. After incubation, it was sub-cultured onto prepared Salmonella shigella agar plates so as to receive individual colonies and further incubated at 37°C for 24 hours. Suspected colonies were streaked onto nutrient agar slant for purification, Gram stain reaction, biochemical and serological identification [20, 21] were caried out on the isolates.

2.6 Antibiotic susceptibility test

Antimicrobial susceptibility of the *Salmonella* isolates was assessed using the Kirby–Bauer disc diffusion technique following Clinical and Laboratory Standards Institute (CLSI) recommendations. The antibiotic agents tested were Amoxicillin–Clavulanate (30 µg), Nalidixic acid (30 µg), Cefotaxime (25 µg), Imipenem (10 µg), Ofloxacin (5 µg), Gentamicin (10 µg), Nitrofurantoin (300 µg), Cefuroxime (30 µg), Ceftriaxone–Sulbactam (45 µg), Ampiclox (10 µg), Cefixime (5 µg), and Levofloxacin (5 µg). A bacterial inoculum was prepared from 24-hour cultures suspended in sterile normal saline and adjusted to the turbidity equivalent of a 0.5 McFarland standard. The standardized suspension was evenly spread across Mueller–Hinton agar plates using sterile swabs. Following a short drying period of approximately 3–5 minutes, antibiotic discs were carefully positioned on the agar surface with sterile forceps, ensuring proper spacing between discs and from the edges of the plates. Each disc was gently pressed to secure full contact with the agar surface. The plates were subsequently incubated at 37°C for 24 hours, after which inhibition zones were measured and interpreted according to established CLSI criteria. Following incubation, the bacterial growth was evaluated and the size of the inhibition zones was determined by measuring from one edge to the other edge of the zone using a metric ruler placed on the plate [22]. Findings of antibiotic resistance testing were recorded as susceptible, intermediate, and resistant [23].

2.7 Determination of Multiple Antibiotic Resistance (MAR) Index

An isolate was considered to exhibit multiple antibiotic resistance when it showed resistance to at least three different antibiotics. The Multiple Antibiotic Resistance (MAR) index was calculated for each isolate to determine the level of resistance using the formula:

$$\mathrm{MAR} = \frac{a}{b}$$

where *a* is number of antibiotics to which the isolate is resistant, and *b* is total number of antibiotics tested for an isolate.

2.8 Molecular Characterization

2.8.1 Salmonella *Genomic DNA Extraction*

Genomic DNA from *Salmonella* isolates was extracted using the Bioneer AccuPrep DNA extraction kit following the manufacturer's instructions. Broth cultures of the isolates were first centrifuged at 8000 rpm for 5 minutes to pellet the bacterial cells, after which the supernatant was removed.

The resulting pellets were resuspended in a solution containing 20 µl Proteinase K and 10 µl RNase and briefly incubated at room temperature to facilitate enzymatic digestion.
Thereafter, 200 µl of Genomic Binding (GB) buffer was added, mixed thoroughly to obtain a uniform suspension, and incubated at 60 °C for 10 minutes to achieve complete cell lysis. Subsequently, 400 µl of chilled absolute ethanol was added, mixed, and the resulting lysate was transferred into a binding column followed by centrifugation at 8000 rpm for 1 minute. The filtrate was discarded, and the column was washed successively with 500 µl of Wash Buffer 1 and Wash Buffer 2, each step followed by centrifugation. To eliminate any remaining ethanol, the column was further centrifuged at 12,000 rpm for 1 minute. DNA was then eluted by adding 200 µl of elution buffer to the column, allowing brief absorption, and centrifuging at 8000 rpm for 1 minute. The purified DNA obtained was collected and stored at 4 °C for short-term preservation.

2.8.2 Polymerase Chain Reaction (PCR) Amplification

Extracted DNA from *Salmonella* isolates was amplified using Polymerase Chain Reaction (PCR) to detect the presence of the **invA** gene. Amplification reactions were prepared using 20 µl of AccuPower PCR pre-mix containing Taq DNA polymerase, dNTPs, and $MgCl_2$. The reaction mixture was completed by adding 16 µl of deionized water, 2 µl of template DNA, and 2 µl each of forward and reverse primers specific for the **invA** gene, followed by gentle centrifugation to ensure proper mixing of the components. A negative control reaction was also included, in which template DNA was replaced with deionized water while maintaining the primer concentration. PCR amplification was conducted under the following thermal cycling conditions: an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles consisting of denaturation at 94 °C for 30 seconds, primer annealing at 52 °C for 30 seconds, and extension at 72 °C for 1 minute. A final extension step at 72 °C for 5 minutes was carried out to complete DNA strand synthesis.

2.8.3 Sample Preparation and Gel Electrophoresis

Agarose gel was prepared by dissolving 3 g of agarose in 100 ml of Tris–acetate–EDTA (TAE) buffer, followed by heating in a microwave until the solution became clear and fully dissolved. The molten agarose was then cooled in a water bath to approximately 50–55 °C, after which 5 µl of ethidium bromide was added and mixed thoroughly. The prepared gel solution was poured into a casting tray equipped with a comb and left at room temperature for 15–30 minutes to allow solidification.
Once the gel solidified, the comb was carefully removed, and the gel was positioned in an electrophoresis tank containing TAE buffer. A 100 bp DNA ladder was loaded into the first well to serve as a molecular size marker, followed by loading of PCR products, while the negative control sample was placed in the final well. Electrophoresis was performed for about 35 minutes, after which the gel was removed and examined under a UV trans-illuminator to visualize and record the DNA bands.

2.8.4 Amplification of the 16S rRNA Gene from Bacterial Isolates

The 16S rRNA gene of the bacterial isolates was amplified using PCR with a total reaction volume of 12.5 µL. The reaction mixture contained Taq 2X Master Mix (New England Biolabs), 1 µL each of 10 µM forward primer (27F: AGAGTTTGATCMT GGCTCAG) and reverse primer (1525R: AAGGAGGTGWTCCARC CGCA), along with 2 µL of template DNA.
Nuclease-free water was added to adjust the mixture to the final reaction volume. Amplification was conducted using standard PCR cycling parameters appropriate for 16S rRNA gene amplification.

2.8.5 Sequencing and Phylogenetic Analysis

Obtained sequence data were analyzed using the Basic Local Alignment Search Tool (BLAST) hosted by the National Center for Biotechnology Information (NCBI) to determine sequence similarity and confirm organism identity. Phylogenetic relationships among the isolates were reconstructed using the Neighbor-Joining algorithm. Evolutionary distances were estimated using the p-distance model, which measures the number of nucleotide differences per site between sequences. All phylogenetic and evolutionary analyses were performed using Molecular Evolutionary Genetics Analysis (MEGA X) software.

2.9 Molecular Antibiotic Resistance and Toxigenic gene Screen

2.9.1 blaTEM and STN Gene Amplification

The *Salmonella* isolates were further subjected to molecular antibiotics resistance and toxigenic gene screening for blaTEM and STN gene using PCR. The primers employed for detection of the blaTEM gene were blaTEM forward (5'-ATGAGTATTCAACATTTCCG-3') and blaTEM reverse (5'-CTGACAGTTACCAATGCTTA-3'). For amplification of the stn gene, the primers used were forward (5′-CTTTGGTCGTAAAATAAGGCG-3′) and reverse (5′-TGCCCAAAGCAGAGAGATTC-3′). PCR amplification reactions were prepared in a final volume of 25 µl containing 12 µl of Taq master mix, 1 µl each of forward and reverse primers, 1 µl of template DNA, and distilled water, with nuclease-free water added to achieve the required final volume. These primers were specifically used to amplify the blaTEM and stn target genes. PCR amplification was conducted in a thermocycler under optimized conditions consisting of an initial denaturation at 94 °C for 1 minute, followed by annealing at 57 °C for 1 minute to facilitate primer attachment, and extension at 72 °C for 10 minutes to enable DNA synthesis by Taq polymerase. A final extension step at 72 °C for 2 minutes ensured completion of DNA strand elongation. The resulting PCR products were resolved by electrophoresis on a 2% agarose gel stained with SYBR® Safe DNA gel stain and run at 100 V and 250 mA for approximately 35 minutes. DNA bands were then visualized under a UV transilluminator to confirm successful amplification and allow further analysis.

3. Results

The antibiotic susceptibility pattern of *Salmonella* isolates recovered from the sampled communities is illustrated in Figure 1. Complete resistance (100%) was observed against cefotaxime, nitrofurantoin, ceftriaxone, and cefixime among all isolates tested. Resistance to nalidixic acid and ofloxacin was recorded in 76% of the isolates, while 88% exhibited resistance to both imipenem and augmentin. A lower resistance rate of 20% was noted for levofloxacin, whereas resistance to cefuroxime, ampiclox, and gentamicin occurred in 92%, 96.2%, and 52% of isolates, respectively. Evaluation of the Multiple Antibiotic Resistance (MAR) index showed that all isolates were resistant to more than two antibiotics, with index values ranging between 0.2 and 0.9, as presented in Figure 2. Agarose gel electrophoresis results demonstrated the presence of the blaTEM gene in all isolates analyzed (Plate 1).

Similarly, amplification of the stn gene produced bands at approximately 260 bp, indicating its occurrence in every isolate screened (Plate 2). Amplification of the 16S rRNA gene is also shown in Plate 2, while Table 1 provides a summary of sequence similarity percentages between the study isolates and reference *Salmonella* strains.

Phylogenetic analysis based on evolutionary distances calculated using the Jukes–Cantor model (Figure 3) confirmed that the obtained 16S rRNA sequences clustered within *Salmonella enterica*. Specifically, isolate RK2 showed 98.19% similarity to *Salmonella enterica* subsp. *enterica* serovar Montevideo strain 11TTUC-046, while RK3 shared 96.83% similarity with *S. enterica* subsp. *enterica* serovar Paratyphi C strain SA49. Isolate RK7 demonstrated 99.51% similarity to *S. enterica* subsp. *enterica* serovar Typhi strain PU4, EU5 displayed 99.26% similarity to *S. enterica* subsp. *enterica* serovar Typhi strain MSAR18, and EU7 exhibited 99.64% similarity to *S. enterica* subsp. *enterica* serovar Enteritidis strain Sa26.

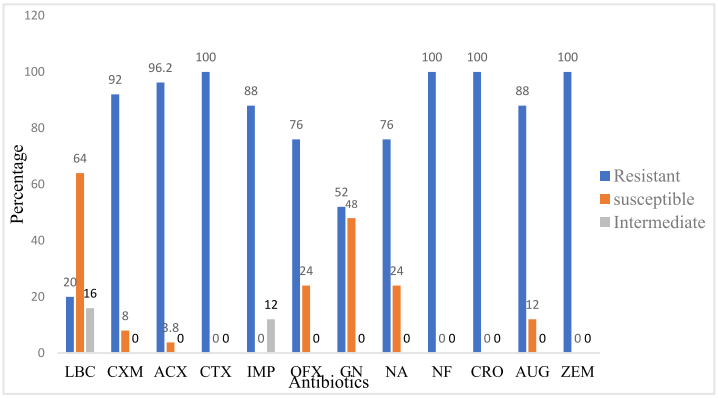


Figure 1: Overall Antibiotic Sensitivity of Salmonella isolates from the sampled locations

Key: LBC= Levofloxacin; GN= Gentamycin; CXM= Cefuroxime; NA= Nalidixic acid; ACX= Ampiclox; NF= Nitrofurantoin; CTX= Cefotaxime; CRO= Ceftriaxone Sulbactam; IMP= Imipenem/cilastacin; AUG= Amoxicillin Clavulanate; OFX= Ofloxacin; ZEM= Cefexime.

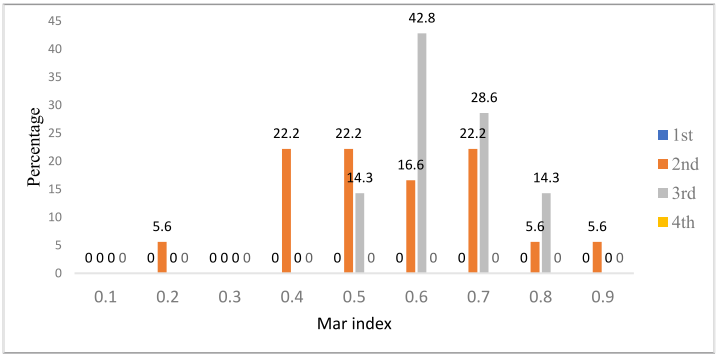
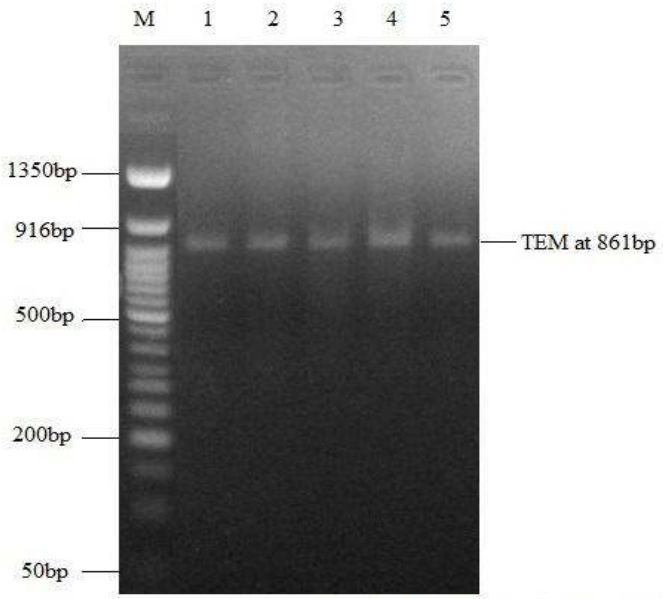
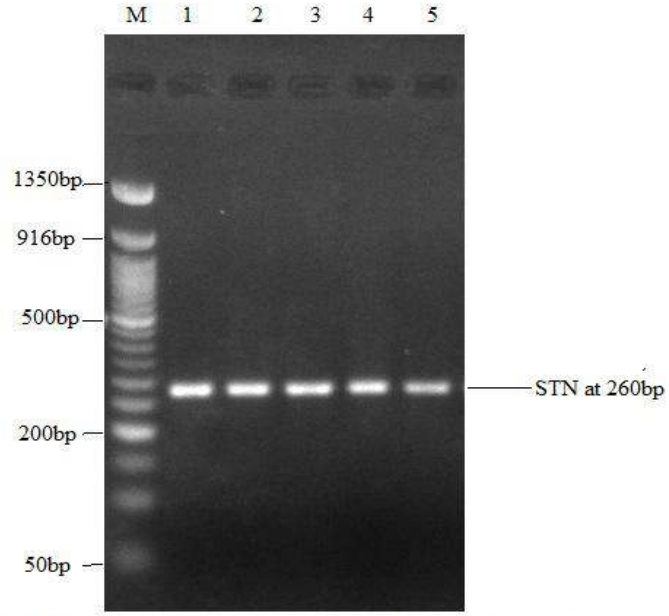


Figure 2: Multiple Antibiotics Resistance Profile of the Isolates Based on period of study



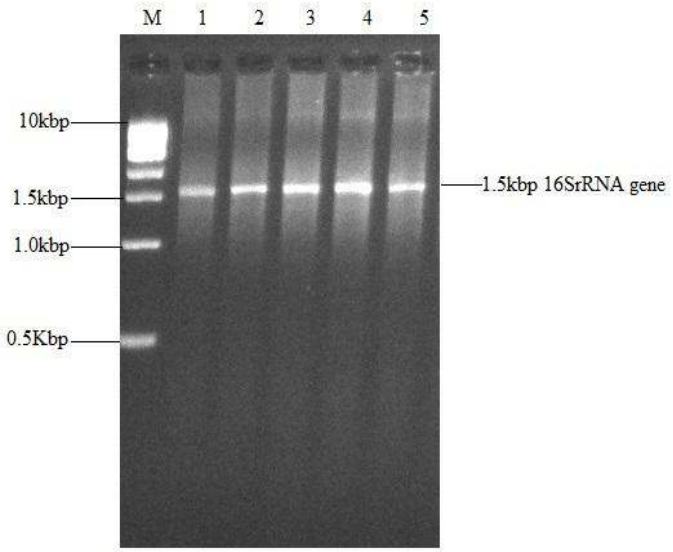
Gel image showing the amplification of TEM at 861bp. Lane 1 = RK2, lane 2 = RK3, lane 3 = RK7, lane 5 = EU5, lane 6 = EU7, lane M is a 50bp DNA

Plate 1: Amplified blaTEM Gene Bands on Agarose Gel after Electrophoresis



Gel image showing the amplification of STN at 260bp. Lane 1 = RK2, lane 2 = RK3, lane 3 = RK7, lane 4 = EU5, lane 5 = EU7, lane M is a 50bp DNA

Plate 2: Amplified STN Gene Bands on Agarose Gel after Electrophoresis



Gel image showing the amplification of the 16SrRNA gene at 1.5Kbp. Lane 1 = RK2, lane 2 = RK3, lane 3 = RK7, lane 4 = EU5, lane 5 = EU7, lane M is a 1kbp DNA ladder

Plate 3: Amplified 16SrRNA gene bands on agarose gel after electrophoresis

Table 1: Molecular Identification of Isolates with Accession Number

S/N	Codes	Description	% Pairwise Identity	Related Accession Number
1	RK2	*Salmonella enterica subsp. enterica serovar Montevideo strain 11TTUC-046*	98.19	CP032816.1
2	RK3	*Salmonella enterica subsp. enterica serovar Paratyphi C strain SA49*	96.83	KU843857.1
3	RK7	*Salmonella enterica subsp. enterica serovar Typhi strain PU4*	99.51	MW029937.1
4	EU5	*Salmonella enterica subsp. enterica serovar Typhi strain MSAR18*	99.26	MZ773245.1
5	EU7	*Salmonella enterica subsp. enterica serovar Enteritidis strain SA26*	99.64	KU843848.1

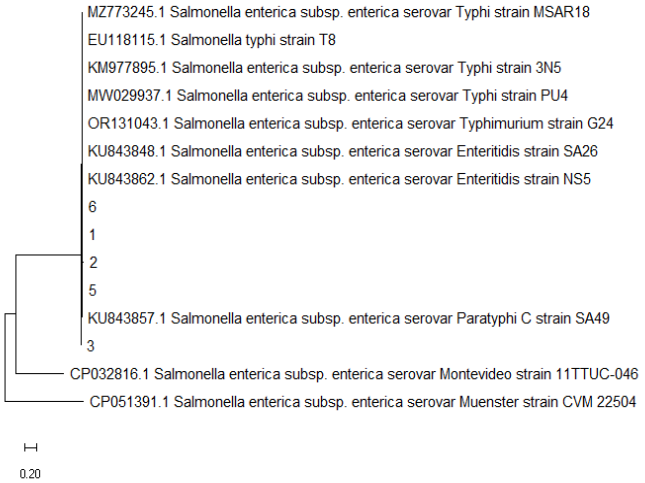


Figure 3: The Phylogenetic tree of the identified organisms

4. Discussion

Evolutionary distances estimated using the Jukes–Cantor model supported the phylogenetic clustering of the obtained 16S rRNA gene sequences within *Salmonella enterica*. Analysis of the 16S rRNA sequences enabled identification of several *Salmonella* strains among the isolates, including *Salmonella enterica* subsp. *enterica* serovar Montevideo strain 11TTUC-046, serovar Paratyphi C strain SA49, serovar Typhi strains PU4 and MSAR18, and serovar Enteritidis strain SA26. The subspecies and serovars identified in this investigation correspond with findings previously reported in related studies [29]. Similarly, Unezem et al. [30] also observed that isolates grouped into a well-defined phylogenetic cluster within *Salmonella enterica* serotypes, supporting the consistency of the present results.

The findings of this study recorded that the highest resistance was observed for antibiotics, Nitrofurantoin, ceftrioaxone, and cefexine in which 100% resistance was recorded in all isolates from the sampled locations while ceftazidime, ampiclox, imipenem, nalidixic, and augmentin showed 75%-100% resistance by the isolates from the sampled location. Of recent, antimicrobial resistance (AMR) has become a big threat to health globally and there is an increase level of antibiotic resistance reported with the globe making it difficult to treat infectious diseases, prolong stay in the hospital could result in increase in cost of medical treatment" [31, 32].

The Multiple Antibiotic Resistance (MAR) index reflects the extent to which a bacterial isolate has been exposed to various antibiotics and serves as a measure of its resistance level. A MAR index value of ≥ 0.2 indicates resistance to multiple antibiotics, and increasing values correspond to resistance against more antibiotics [33]. All of the tested *Salmonella* isolates showed multidrug-resistance with MAR index 0.25 to 1.0. The results of this study are consistent with those reported by Alexander et al. [34]. in which the MAR index of the *Staphyloccoccus* isolates ranged from 0.2 to 1.0. This suggests that the isolates exhibited resistance to most of the antibiotics tested, which may be due to the existence of multiple resistance genes within their genomes that confer antimicrobial resistance.

The STN gene and *BLAtem* gene were detected in all (5) five isolates of *Salmonella* isolated. The 100% detection rate of the gene indicates that it is highly conserved among the *Salmonella enterica* isolates. The STN gene is recognized as a virulence factor in *Salmonella* and has been implicated as a potential contributor to diarrheal disease, as previous studies have demonstrated its enterotoxic activity. The finding in this study revealed that 100% of the predominant *Salmonella* serovar displayed an MDR phenotype which is evident in the MAR index analysis. The prevalence of multidrug resistance recorded in this study can be likely linked with resistance gene such as blaTEM as shown in the result of the antibiotic sensitivity test and the MAR index results.

5. Conclusion

The study has indicated a high level of antibiotic resistance among bacterial isolates from Rumuokparali, Elelenwo, and Eliozu communities. Isolates from all three locations exhibited significant resistance to multiple antibiotics, with 100% resistance observed against key drugs such as nitrofurantoin, ceftriaxone, augmentin, cefexime, and cefotaxime in several locations, the temporal distribution has shown that *Salmonella* isolates with high MAR indices were predominantly detected in the second and third quarters. Resistance to these antibiotic groups must have been brought about by presence the of the blaTEM gene in the species which was probably transmitted to the vast number of these species in a bid to help them thrive in the presence of these antibiotic classes. The study confirms the presence of *STN* genes in all molecularly tested isolates, which is a key indication of conserved virulence and resistance traits among *Salmonella enterica* strains in the region's water supply.

Competing Interests

Authors have declared that no competing interests exist.

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