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The role of 16S rRNA sequencing in bridging phenotypic-genotypic gaps in *Salmonella* identification: Evidence from a Nigerian endemic zone

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Abstract:

Background: Accurate laboratory identification of *Salmonella* species is essential for effective diagnosis, antimicrobial stewardship, and surveillance of enteric fever. Conventional culture and biochemical methods remain the primary diagnostic approach in many laboratories in resource-limited countries but are prone to misidentification because several members of the family Enterobacteriaceae exhibit similar phenotypic characteristics. This study evaluated the role of 16S rRNA gene sequencing in validating phenotypic identification of *Salmonella* isolates obtained from patients with suspected enteric fever in Nigeria.

Methodology: Blood samples collected from a total of 425 patients with suspected enteric fever were cultured using standard bacteriological procedures. Presumptive *Salmonella* isolates were identified by conventional biochemical tests, including triple sugar iron (TSI) agar reactions, urease, citrate, motility, and indole tests. Genomic DNA was extracted from all presumptive isolates, and bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) and sequenced by Sanger method. Sequence identities were determined using the National Center for Biotechnology Information (NCBI) BLAST database, while phylogenetic relationships were inferred using the Maximum Likelihood method with bootstrap analysis.

Results: Conventional phenotypic methods identified 32 (7.5%) isolates as *Salmonella* from the patients. However, sequencing confirmed only 25 (78.1% of presumptive isolates and 5.9% of blood cultures as *Salmonella*, while 7 (21.9%) were reclassified as non-*Salmonella* Enterobacteriaceae, including *Enterobacter oligotrophicus* (n=3), *Enterobacter hormaechei* (n=1), *Citrobacter freundii* (n=1), *Citrobacter youngae* (n=1), and *Klebsiella oxytoca* (n=1). Using sequencing as the reference standard, phenotypic identification demonstrated 100% sensitivity (95% CI: 84.6–100.0), 81.6% specificity (95% CI: 66.6–91.6), positive predictive value of 78.1% (95% CI: 60.0–90.7), and negative predictive value of 100% (95% CI: 88.8–100.0), with substantial agreement (Cohen's $\kappa = 0.78$, $p < 0.001$). Among the confirmed isolates, *S. Typhimurium* (n=11) was predominant, followed by untyped *S. enterica* (n=6), and single isolates of *S. Enteritidis*, *S. Heidelberg*, *S. Infantis*, *S. Typhi*, *S. Livingstone*, *S. Paratyphi B*, *S. diarizonae*, and *S. bongori*. Phylogenetic reconstruction grouped these isolates into four major clades with strong bootstrap support, confirming distinct evolutionary lineages within the circulating population.

Conclusion: Conventional phenotypic methods substantially overestimated the prevalence of *Salmonella* infection because of bacterial misidentification. Integration of 16S rRNA gene sequencing into diagnostic workflows significantly improved species confirmation, refined prevalence estimates, and provided valuable phylogenetic information that can strengthen antimicrobial resistance surveillance and public health decision-making in endemic settings.

Keywords: *Salmonella*; 16S rRNA; Sanger sequencing; Enteric fever; Blood culture; Phylogenetic analysis

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Rôle du séquençage de l'ARNr 16S dans la résolution des discordances phénotypiques et génotypiques pour l'identification de *Salmonella*: données issues d'une zone endémique du Nigéria

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Résumé:

Contexte: L'identification précise des espèces de *Salmonella* en laboratoire est essentielle pour un diagnostic efficace, une gestion optimale des antimicrobiens et la surveillance de la fièvre typhoïde. Les méthodes conventionnelles de culture et de biochimie restent la principale approche diagnostique dans de nombreux laboratoires des pays à ressources limitées, mais elles sont sujettes à des erreurs d'identification, car plusieurs membres de la famille des Enterobacteriaceae présentent des caractéristiques phénotypiques similaires. Cette étude a évalué le rôle du séquençage du gène de l'ARNr 16S dans la validation de l'identification phénotypique des isolats de *Salmonella* obtenus chez des patients présentant une suspicion de fièvre typhoïde au Nigéria.

Méthodologie: Des échantillons de sang prélevés chez un total de 425 patients présentant une suspicion de fièvre typhoïde ont été mis en culture selon les procédures bactériologiques standard. Les isolats présumés de *Salmonella* ont été identifiés par des tests biochimiques conventionnels, notamment les réactions sur gélose TSI (Triple Sugar Iron), les tests à l'uréase, au citrate, de mobilité et d'indole. L'ADN génomique a été extrait de tous les isolats présumés, et le gène de l'ARNr 16S bactérien a été amplifié par réaction en chaîne par polymérase (PCR) et séquencé par la méthode de Sanger. Les identités de séquences ont été déterminées à l'aide de la base de données BLAST du NCBI (Centre National d'Information sur la Biotechnologie), tandis que les relations phylogénétiques ont été inférées par la méthode du maximum de vraisemblance avec analyse de bootstrap.

Résultats: Les méthodes phénotypiques conventionnelles ont identifié 32 isolats (7,5%) comme étant des *Salmonella* chez les patients. Cependant, le séquençage n'a confirmé que 25 isolats (78,1% des isolats présomptifs et 5,9% des hémocultures) comme étant des *Salmonella*, tandis que 7 (21,9%) ont été reclassés comme Enterobacteriaceae non-*Salmonella*, notamment *Enterobacter oligotrophicus* (n=3), *Enterobacter hormaechei* (n=1), *Citrobacter freundii* (n=1), *Citrobacter youngae* (n=1) et *Klebsiella oxytoca* (n=1). En utilisant le séquençage comme méthode de référence, l'identification phénotypique a démontré une sensibilité de 100% (IC à 95%: 84,6–100,0), une spécificité de 81,6% (IC à 95%: 66,6–91,6), une valeur prédictive positive de 78,1% (IC à 95%: 60,0–90,7) et une valeur prédictive négative de 100% (IC à 95%: 88,8–100,0), avec une concordance substantielle (κ de Cohen=0,78, $p<0,001$). Parmi les isolats confirmés, *S. Typhimurium* (n=11) était prédominant, suivi de *S. enterica* non typé (n=6), et d'isolats uniques de *S. Enteritidis*, *S. Heidelberg*, *S. Infantis*, *S. Typhi*, *S. Livingstone*, *S. Paratyphi B*, *S. diarizonae* et *S. bongori*. La reconstruction phylogénétique a regroupé ces isolats en quatre clades principaux avec un fort soutien bootstrap, confirmant des lignées évolutives distinctes au sein de la population circulante.

Conclusion: Les méthodes phénotypiques conventionnelles surestimaient considérablement la prévalence de l'infection à *Salmonella* en raison d'erreurs d'identification bactérienne. L'intégration du séquençage du gène de l'ARNr 16S dans les flux de travail diagnostiques a significativement amélioré la confirmation des espèces, affiné les estimations de prévalence et fourni des informations phylogénétiques précieuses qui peuvent renforcer la surveillance de la résistance aux antimicrobiens et la prise de décision en matière de santé publique contexte endémique.

Mots-clés: *Salmonella*; ARNr 16S; séquençage Sanger; fièvre typhoïde; hémoculture; analyse phylogénétique

Introduction:

Enteric fever remains a major public health challenge in many low- and middle-income countries, particularly in sub-Saharan Africa and South Asia, where inadequate sanitation, unsafe drinking water, rapid urbanization, and limited access to quality healthcare facilitate transmission of *Salmonella enterica* serovars Typhi and Paratyphi. Despite global reductions in disease burden following improvements in water, sanitation, and vaccination programmes, millions of new infections and

thousands of deaths continue to occur annually, with Africa accounting for a substantial proportion of the burden. Recent global estimates indicate that enteric fever remains an important cause of morbidity and mortality, particularly among children and young adults living in endemic settings (1-5).

In Nigeria, enteric fever continues to be one of the most frequently diagnosed causes of prolonged febrile illness. The epidemiology of the disease is influenced by poor environmental sanitation, inadequate potable water supply, indiscriminate antimicrobial use, and

weak laboratory diagnostic capacity. Although clinical diagnosis is common, the nonspecific presentation of enteric fever overlaps considerably with malaria, invasive bacterial infections, and other febrile illnesses, making laboratory confirmation essential for appropriate patient management (4,6–8).

Blood culture remains the accepted reference standard for laboratory diagnosis of enteric fever because it provides viable isolates for antimicrobial susceptibility testing and epidemiological surveillance. However, the sensitivity of blood culture is limited by several factors, including low bacterial concentration in blood, prior antimicrobial exposure, delayed presentation to healthcare facilities, and inadequate laboratory infrastructure. Consequently, many diagnostic laboratories in resource-limited settings rely heavily on conventional biochemical identification following culture isolation (9–11).

Routine phenotypic identification of *Salmonella* species is based primarily on colony morphology, carbohydrate fermentation patterns, hydrogen sulfide production, motility, citrate utilization, urease activity, lysine decarboxylation, and triple sugar iron (TSI) agar reactions. Although these methods are inexpensive and widely available, several members of the family *Enterobacteriaceae*, including *Citrobacter*, *Enterobacter*, *Klebsiella*, and related genera, may exhibit similar biochemical characteristics. Such phenotypic overlap frequently results in false-positive identification of *Salmonella*, leading to inaccurate diagnosis, inappropriate antimicrobial therapy, and distorted surveillance data (12–15).

The introduction of molecular identification methods has substantially improved the accuracy of bacterial taxonomy. Among these, sequencing of the 16S ribosomal RNA (16S rRNA) gene has become one of the most widely used approaches for bacterial identification because the gene contains highly conserved regions suitable for universal amplification as well as variable regions that permit differentiation among bacterial taxa. Comparison of 16S rRNA gene sequences with curated international databases enables reliable identification of bacterial isolates that cannot be confidently resolved using conventional biochemical methods alone (16–18).

Beyond species identification, 16S rRNA sequencing also provides valuable phylogenetic information by elucidating evolutionary relationships among bacterial isolates. Although the technique lacks sufficient discriminatory power for detailed serovar differentiation compared with whole-genome sequencing, it remains an

important molecular tool for confirming bacterial identity, validating phenotypic diagnoses, and supporting epidemiological investigations, particularly in laboratories where advanced genomic technologies are not readily available (17,19,20).

Accurate laboratory identification of *Salmonella* has become increasingly important in the era of antimicrobial resistance. Misidentification of non-*Salmonella* Enterobacteriaceae as *Salmonella* may result in erroneous estimates of disease prevalence, inaccurate antimicrobial resistance surveillance, and inappropriate public health interventions. Integration of molecular confirmation into routine diagnostic workflows therefore represents an important strategy for improving surveillance quality and guiding evidence-based antimicrobial stewardship programmes (3,21–24).

This study evaluated the performance of conventional phenotypic identification methods compared with 16S rRNA gene sequencing for the identification of *Salmonella* isolates recovered from patients with suspected enteric fever attending selected healthcare facilities in Nigeria. Specifically, the study determined the prevalence of culture-confirmed *Salmonella*, assessed the diagnostic performance of routine biochemical identification relative to molecular confirmation, characterized organisms responsible for phenotypic misclassification, and evaluated the phylogenetic relationships among molecularly confirmed *Salmonella* isolates. The findings provide evidence for strengthening laboratory diagnosis and improving surveillance of enteric fever in resource-limited settings.

Materials and method:

Study design and setting:

This study employed a hospital-based cross-sectional laboratory investigation to evaluate the performance of conventional phenotypic identification methods relative to 16S rRNA gene sequencing for the identification of *Salmonella* isolates recovered from blood cultures of patients with suspected enteric fever. This study was conducted in Keffi Local Government Area (LGA), Nasarawa State, Nigeria. Keffi is a peri-urban setting with a population characterized by rapid growth, limited water and sanitation infrastructure, and high burdens of communicable diseases.

The study site was selected due to its endemicity for enteric fever and the availability of secondary and tertiary healthcare facilities that manage large volumes of febrile patients. The study was carried out over a defined surveillance period of six months, coinciding

with both rainy and dry seasons to capture temporal variation in infection risk.

Study participant recruitment:

Patients presenting to selected health facilities with febrile illness were screened for eligibility. Inclusion criteria were; (i) age 1–65 years, (ii) fever $\geq 38^\circ\text{C}$ persisting for more than three days, and (iii) clinical features and complications suspicion of enteric fever by attending clinicians. These clinical features and complications included abdominal pain, diarrhoea or constipation, abdominal swelling, anorexia, vomiting, furring of the tongue, headache, bone pains, fatigability, pallor, meningeal irritation signs, rash, convulsion, and altered consciousness.

Exclusion criteria included prior antibiotic therapy within 72 hours, known chronic illness (e.g., HIV, TB), or refusal to consent. A total of 425 consecutive patients meeting inclusion criteria were enrolled, reflecting the typical patient load for febrile illnesses in the region. Informed consent was obtained from adult participants or guardians of minors, with ethical approval granted by the FMCK Health Research Ethics Committee.

Ethical consideration:

Ethical approval was obtained from the FMC Keffi Health Research Ethics Committee. All study procedures complied with the Declaration of Helsinki and Nigerian National Ethics guidelines. Participation was voluntary, and confidentiality was maintained.

Data collection:

A structured, interviewer-administered questionnaire captured demographic and clinical data. Data collectors were trained research assistants who conducted interviews in English or local languages. The questionnaire was pre-tested for reliability and adapted from validated tools used in similar epidemiological studies (26,27).

Diagnostic workflow for phenotypic and molecular identification of *Salmonella*:

A structured diagnostic workflow was applied, integrating routine phenotypic testing with molecular confirmation. The workflow illustrates the progression from blood culture and biochemical screening to molecular verification and highlights stages where phenotypic misclassification occurred (Fig 1). Fig 2 showed the diagnostic algorithm illustrating phenotypic-to-molecular confirmation of *Salmonella* identification.

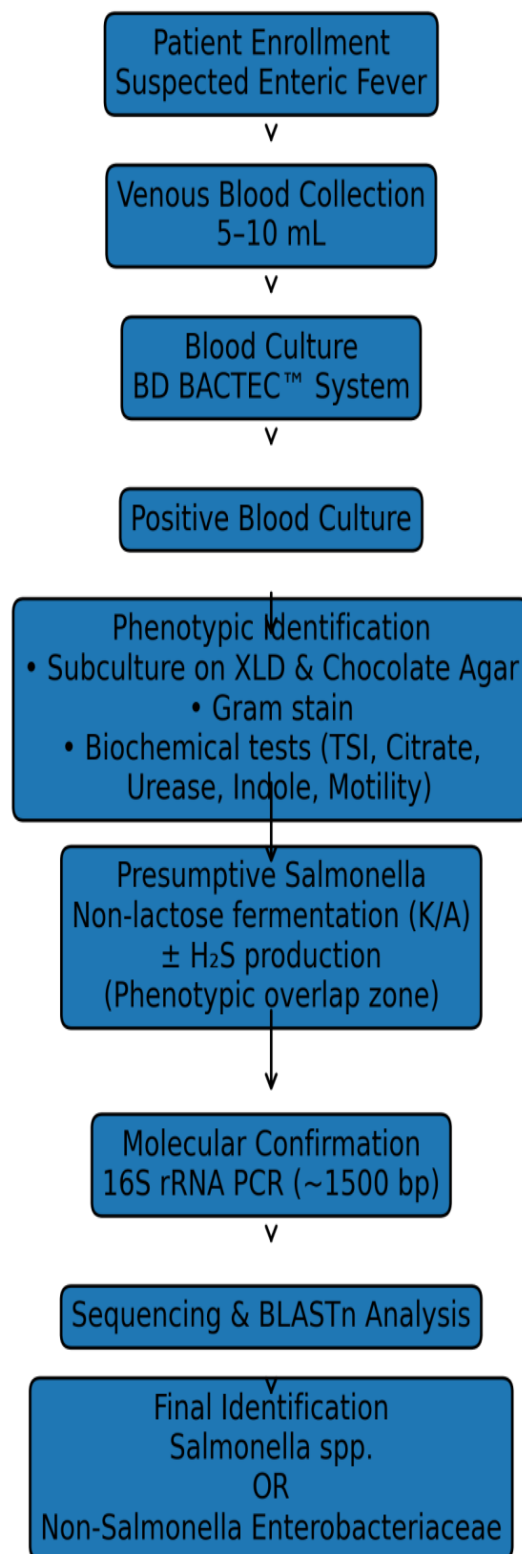


Fig 1: Workflow of phenotypic and molecular identification of *Salmonella*

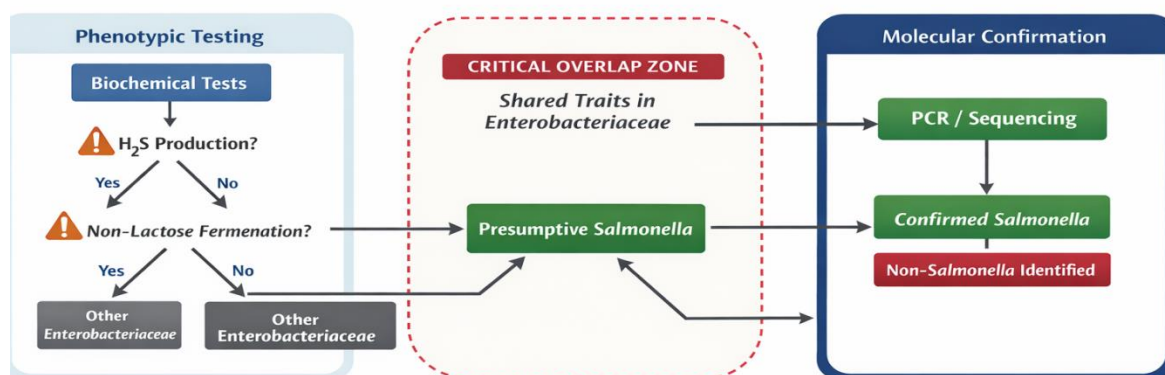


Fig 2: Diagnostic algorithm illustrating phenotypic-to-molecular confirmation of *Salmonella* identification

Stepwise diagnostic algorithm depicting the workflow for identification of presumptive *Salmonella* isolates. Initial classification was based on conventional phenotypic testing, including biochemical reactions indicative of non-lactose fermentation and hydrogen sulfide (H_2S) production. Due to overlapping phenotypic traits among members of the family Enterobacteriaceae, some non-*Salmonella* isolates (e. g. *Enterobacter*, *Klebsiella*, and *Citrobacter* spp) met these criteria and entered a critical overlap zone. Molecular confirmation by 16S rRNA gene PCR and sequencing enabled definitive species-level identification, distinguishing true *Salmonella* isolates from phenotypic false positives.

Blood culture and phenotypic identification:

For each participant, 5–10 mL of venous blood was collected aseptically and inoculated into BD BACTEC™ blood culture bottles. Cultures were incubated in automated BACTEC systems and monitored for growth for up to seven days. Positive bottles were sub-cultured onto chocolate blood agar and xylose lysine deoxycholate (XLD) agar.

Colonies exhibiting typical *Salmonella*-like characteristics were further evaluated by Gram staining and standard biochemical tests (Triple Sugar Iron (TSI) agar reaction, MR-VP, Urease test, Citrate utilization test, Motility test, and Indole test). Phenotypic identification was performed according to the Clinical and Laboratory Standards Institute guidelines (14) by trained microbiologists.

16S rRNA gene amplification and sequencing:

All isolates presumptively identified as *Salmonella* by phenotypic methods were subjected to molecular confirmation using 16S rRNA sequencing. DNA was extracted using a boiling method followed by ethanol precipitation. The universal primers 27F (5'-AGAGTTT GATCMTGGCTCAG-3') and 1492R (5'-TACGGY TACCTTGTTACGACTT-3') were used to amplify approximately 1,500 base pairs of the 16S rRNA gene. PCR reactions were carried out in 25 μ L volumes with DreamTaq Green Master Mix (Thermo Scientific), using an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C (30 seconds), annealing at 55°C (30 seconds), extension at 72°C (90 seconds), and a final elongation at 72°C for 10 minutes. Amplicons were visuali-

zed on 1.5% agarose gels stained with ethidium bromide.

The PCR products were purified (Invitrogen PureLink PCR Purification Kit, Invitrogen/Thermo Fisher Scientific, Baltics UAB, Vilnius, Lithuania) and sequenced bidirectionally by the Sanger sequencing (Macrogen, South Korea). Raw sequences were assembled and trimmed using BioEdit v7.2, and consensus sequences were compared to reference sequences in the NCBI GenBank database using BLASTn. Sequences with $\geq 99\%$ identity and coverage were considered definitive matches for species-level classification (16,17).

Phylogenetic analysis:

To contextualize the genetic relatedness of confirmed *Salmonella* isolates, representative 16S rRNA gene sequences were aligned with global reference strains retrieved from GenBank. Multiple sequence alignment was performed using ClustalW in MEGA v11, and phylogenetic trees were constructed using the Maximum Likelihood method with 1,000 bootstrap replicates (24,25). Trees were rooted with *Escherichia coli* K-12 as an outgroup to confirm evolutionary placement (21,24). Bootstrap values $\geq 70\%$ were considered to indicate strong phylogenetic support (25).

Quality assurance:

Laboratory procedures adhered to standard biosafety practices. Positive and negative controls were included in each PCR batch. To minimize cross-contamination, DNA extraction, PCR setup, and post-amplification handling were conducted in separate rooms with dedicated pipettes. Sequences were checked for contami-

nation by comparing against both forward and reverse primers and excluding chimeric sequences.

Data analysis:

Data were analyzed using SPSS version 26.0. Participant characteristics were summarized using descriptive statistics, with categorical variables presented as frequencies and percentages and continuous variables as means $\pm$ standard deviations. Concordance between phenotypic identification and molecular confirmation was assessed using 2 $\times$ 2 contingency tables, with 16S rRNA gene sequencing as the reference standard.

Diagnostic performance of phenotypic methods was evaluated by calculating sensitivity, specificity, positive and negative predictive values with 95% confidence intervals. Agreement beyond chance between phenotypic identification and molecular confirmation was quantified using Cohen's kappa (κ) statistic. Misclassified isolates and patterns of phenotypic overlap were summarized descriptively, and phylogenetic relationships among confirmed *Salmonella* isolates were assessed using 16S rRNA gene sequence-based analysis. A *p*-value < 0.05 was considered statistically significant.

Results:

Participant characteristics:

A total of 425 patients with suspected

enteric fever were enrolled. The mean age was 22.1 $\pm$ 14.4 years (range: 1–65 years), and females accounted for 54.6% (232/425) of participants. All patients presented with febrile illness lasting more than three days. Demographic characteristics are summarized in Table 1.

Blood culture yield and phenotypic identification:

Of the blood cultures processed for 425 selected patients, 63 (14.8%) yielded bacterial growth, giving a microbiologically documented prevalence of 14.8%. Based on routine phenotypic identification using selective media and biochemical assays, 32 isolates (50.8% of culture-positive samples and 7.5% of blood cultures) were identified as *Salmonella* spp while 31 isolates were identified as non-*Salmonella* pathogens.

Molecular confirmation by 16S rRNA gene amplification and sequencing:

All 32 phenotypically identified *Salmonella* isolates subjected to 16S rRNA gene amplification and sequencing, amplified to the nearly full-length 16S rRNA gene (~1,500 bp), as shown in agarose gel electrophoresis (Fig 3). Molecular analysis confirmed 25 isolates (78.1%) as *Salmonella* spp with $\geq$ 99% sequence identity, while 7 isolates (21.9%) were reclassified as non-*Salmonella* Enterobacteriaceae.

Table 1: Demographic characteristics of study participants

General characteristics	Sex		Total (n=425)	χ²	p-value
	Male (n=193)	Female (n=232)			
Age group (years)					
1-10	66 (34.2)	52 (22.4)	118 (27.8)	20.737	0.001
11-20	41 (21.2)	50 (21.6)	91 (21.4)		
21-30	33 (17.1)	79 (34.1)	112 (26.4)		
31-40	28 (14.5)	33 (14.2)	61 (14.4)		
41-50	14 (7.3)	13 (5.6)	27 (6.4)		
>50	11 (5.7)	5 (2.2)	16 (3.8)		
Mean age (± SD)	21.66±16.22	22.50±12.67	22.12±14.38	0.597	0.551

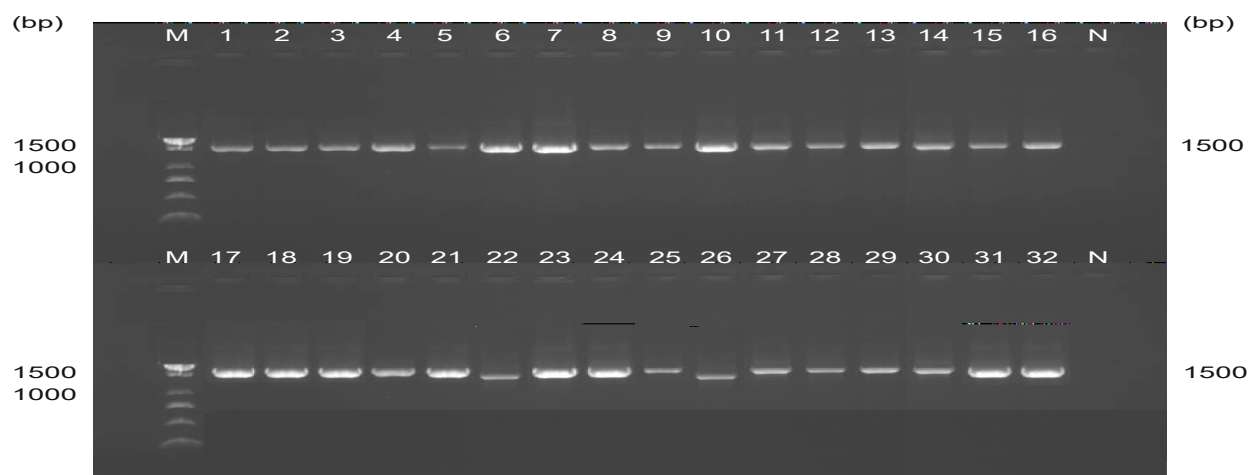


Fig 3: Agarose gel electrophoresis of 16S rRNA gene PCR products from phenotypic *Salmonella* isolates

Representative 1.5% agarose gel showing PCR amplification of the nearly full-length 16S rRNA gene (~1,500 bp) from 32 isolates initially identified as *Salmonella* by phenotypic methods using universal primers 27F and 1492R. Lanes 1–32 represent PCR amplicons from individual isolates. Lane M contains a 250 bp DNA ladder (Promega), and lane N represents the negative control, which showed no amplification.

Table 2: Concordance between phenotypic and molecular identification of blood culture isolates

Phenotypic identification	Molecular confirmed <i>Salmonella</i>	Molecular-confirmed Non- <i>Salmonella</i>	Total
<i>Salmonella</i>	25 (True positives)	7 (False positives)	32
Non- <i>Salmonella</i>	0 (False negatives)	31 (True negatives)	31
Total	25	38	63

Note: Cohen's kappa coefficient (κ) = 0.78 ($p < .001$), indicating substantial agreement between phenotypic and molecular identification methods.

Phenotypic–genotypic concordance and diagnostic performance:

Comparison of phenotypic identification with molecular confirmation via 16S rRNA gene sequencing, as summarized in Table 2, revealed substantial agreement between the two diagnostic approaches (Cohen's $\kappa=0.78$, $p<0.001$). Among 32 isolates initially classified as *Salmonella* by conventional phenotypic methods, 25 (78.1%) were confirmed as true *Salmonella*. However, 7 isolates (21.9%) were identified as false positive *Salmonella*, representing non-*Salmonella* Enterobacteriaceae that were misclassified due to overlapping biochemical traits.

Importantly, no false-negative phenotypic identifications were observed; all 31 isolates initially categorized as non-*Salmonella* by routine testing were unequivocally confirmed as true negatives by sequencing. These findings indicate that while phenotypic screening is highly reliable for excluding *Salmonella* (NPV=100%), its imperfect specificity results in clinically meaningful overestimation of the disease prevalence, reinforcing the necessity of molecular confirmation for definitive species-level identification.

Table 3: Diagnostic performance of phenotypic identification of *Salmonella* compared to 16S rRNA sequencing

Parameter	Estimate (%)	95% Confidence Interval (Clopper-Pearson)
Sensitivity	100.0	84.6 – 100.0
Specificity	81.6	66.6 – 91.6
PPV	78.1	60.0 – 90.7
NPV	100.0	88.8 – 100.0

Reference standard: 16S rRNA gene sequencing
Sample size: n = 63 bloodstream isolates
PPV = Positive Predictive Value
NPV=Negative Predictive Value

Using 16S rRNA gene sequencing as the reference standard, the diagnostic performance of phenotypic identification for *Salmonella* was comprehensively evaluated (Table 3). The method demonstrated sensitivity of 100.0% (95% CI: 84.6–100.0), confirming that no culture-positive *Salmonella* case was missed by conventional biochemical testing, a reflection of the zero false-negative results observed in our concordance analysis. The specificity was 81.6% (95% CI: 66.6–91.6), indicating that while phenotypic tests correctly excluded majority of non-*Salmonella* isolates, they

still misclassified 7 of 38 molecular-confirmed non-*Salmonella* Enterobacteriaceae as false positives. Accordingly, the positive predictive value (PPV) was 78.1% (95% CI: 60.0–90.7) meaning that approximately one in five presumptive *Salmonella* isolates identified by routine methods would ultimately require molecular correction. In contrast, the negative predictive value (NPV) was 100.0% (95% CI: 88.8–100.0), reinforcing that a negative phenotypic result is entirely conclusive and reliably rules out *Salmonella* bacteremia.

Collectively, these recalculated metrics correcting the earlier overestimation of false positives and underestimation of specificity highlight that while phenotypic screening serves as a highly sensitive initial tool with an excellent rule-out capacity; its imperfect specificity creates a clinically meaningful risk of overdiagnosis. This underscores the critical need for molecular confirmation to guide appropriate antimicrobial therapy and accurate disease surveillance in endemic settings. The extent of phenotypic misclassification and molecular confirmation is summarized graphically in Fig 4, illustrating the proportion of true-positive and false-positive *Salmonella* identifications.

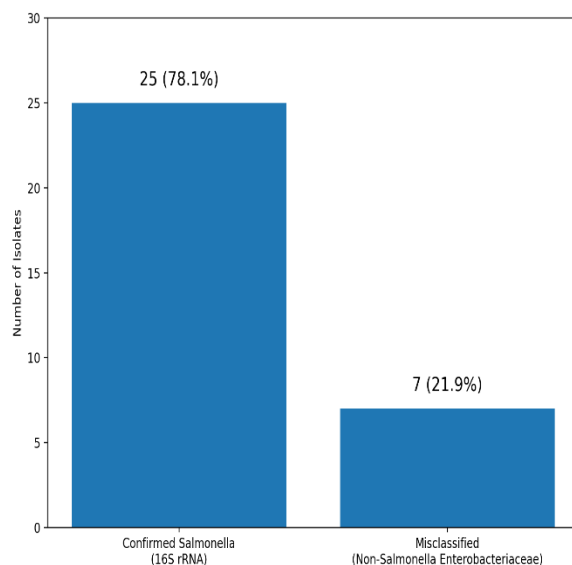


Fig 4: Phenotypic-genotypic concordance of presumptive *Salmonella* isolates

Misclassified isolates and patterns of phenotypic overlap:

The 16S rRNA gene sequence analysis demonstrated that only 25 of the 32 presumptive *Salmonella* isolates were correctly identified, corresponding to molecular prevalence of 5.9% among all clinical specimens. The remaining 7 isolates initially classified as *Salmonella* by phenotypic methods were misidentified and were found to belong to other members of the family Enterobacteriaceae, indicating that conventional phenotypic methods had overestimated the prevalence of *Salmonella* infection.

The confirmed *Salmonella* isolates comprised 11 isolates of *Salmonella* Typhimurium, 6 isolates identified as *Salmonella enterica* without further serovar assignment, and 1 isolate each of *Salmonella* Enteritidis, *Salmonella* Heidelberg, *Salmonella* Infantis, *Salmonella* Typhi, *Salmonella* Livingstone, *Salmonella* Paratyphi B, *Salmonella* diarizonae, and *Salmonella* bongori (Table 4). The 7 phenotypically misidentified isolates were characterized by molecular method as 3 *Enterobacter oligotrophicus*, 1 *Enterobacter hormaechei*, 1 *Citrobacter freundii*, 1 *Citrobacter youngae*, and 1 *Klebsiella oxytoca*. These findings demonstrate the inability of phenotypic identification alone to reliably distinguish *Salmonella* from closely related members of the family Enterobacteriaceae (Table 5).

Table 6 presents a side-by-side comparison of molecular identifications and key biochemical reactions for all 32 isolates initially presumptively identified as *Salmonella* by phenotypic methods. Among these, molecular confirmation by 16S rRNA gene sequencing established that 25 isolates (78.1%) were genuine *Salmonella* species, while 7 isolates (21.9%) were reclassified as other members of the Enterobacteriaceae. Strikingly, all 25 confirmed *Salmonella* isolates comprising diverse serovars including *S. Typhimurium*, *S. enterica* (untyped), *S. Enteritidis*, *S. Typhi*, *S. Paratyphi B*, *S. Infantis*, *S. Livingstone*, *S. Heidelberg*, *S. diarizonae*, and *S. bongori* exhibited the classic biochemical profile of non-lactose fermentation (K/A reaction on TSI agar) and hydrogen sulfide (H₂S) production. Consequently, all were categorized under the "Typical *Salmonella*" phenotypic interpretation, underscoring that the conventional biochemical algorithm performed perfectly for this subset of true positives.

Table 4: Molecular identification of presumptive *Salmonella* isolates by 16S rRNA sequencing

Molecular identification	Number of isolates	Percentage of confirmed isolates (%)
<i>Salmonella</i> Typhimurium	11	44.0
<i>Salmonella enterica</i> (untyped)	6	24.0
<i>Salmonella</i> Enteritidis	1	4.0
<i>Salmonella</i> Heidelberg	1	4.0
<i>Salmonella</i> Infantis	1	4.0
<i>Salmonella</i> Typhi	1	4.0
<i>Salmonella</i> Livingstone	1	4.0
<i>Salmonella</i> Paratyphi B	1	4.0
<i>Salmonella</i> diarizonae	1	4.0
<i>Salmonella</i> bongori	1	4.0
Total confirmed <i>Salmonella</i>	25	100.0

Table 5: Non-*Salmonella* Enterobacteriaceae isolates misidentified as *Salmonella* by phenotypic methods and reclassified by 16S rRNA gene sequencing

Phenotypic identification	True identity (by 16S rRNA sequencing)	Number
<i>Salmonella</i> spp	<i>Enterobacter Oligotrophicus</i>	3
<i>Salmonella</i> spp	<i>Enterobacter hormaechei</i>	1
<i>Salmonella</i> spp	<i>Citrobacter freundii</i>	1
<i>Salmonella</i> spp	<i>Citrobacter youngae</i>	1
<i>Salmonella</i> spp	<i>Klebsiella oxytoca</i>	1
Total		7

In contrast, the 7 misidentified non-*Salmonella* isolates demonstrated heterogeneous biochemical profiles that accounted for their diagnostic confusion. Three of these *Citrobacter freundii* (S7), *Klebsiella oxytoca* (S27), and *Enterobacter hormaechei* (S32) were particularly deceptive, as they not only fermented non-lactose (K/A) but also produced hydrogen sulfide (H₂S+), thereby creating a complete phenotypic mimicry of *Salmonella* and placing them in the "high-risk misidentification" category. The remaining 4 false positives *Citrobacter youngae* (S22) and 3 *Enterobacter oligotrophicus* isolates (S28–S30) were H₂S-negative (H₂S–) yet were still misclassified as *Salmonella*, suggesting that other overlapping biochemical traits, such as urease activity, citrate utilization, or motility patterns, may have been interpreted ambiguously or overlooked during routine screening.

Collectively, these findings demonstrate that while the combination of non-lactose fermentation and H₂S production reliably iden-

tified true *Salmonella* in this cohort, the occurrence of H₂S-producing non-*Salmonella* organisms represents a significant diagnostic pitfall, and conventional phenotypic algorithms cannot be solely relied upon for definitive species-level identification without molecular corroboration.

Phylogenetic validation of confirmed *Salmonella* isolates:

Phylogenetic reconstruction based on partial 16S rRNA gene sequences demonstrated considerable genetic diversity among the confirmed *Salmonella* isolates while maintaining clear clustering within the genus. Four principal clades were identified (Figs 5 and 6).

The first and largest clade consisted predominantly of *S. Typhimurium* isolates together with several *Salmonella enterica* strains, indicating close evolutionary relatedness among these organisms. Additional clades contained *S. Enteritidis*, *S. Heidelberg*, *S. Infantis*, *S. Livingstone*, and *S. bongori*, while *S. Typhi* and *S. Paratyphi B* clustered within a strongly supported basal lineage.

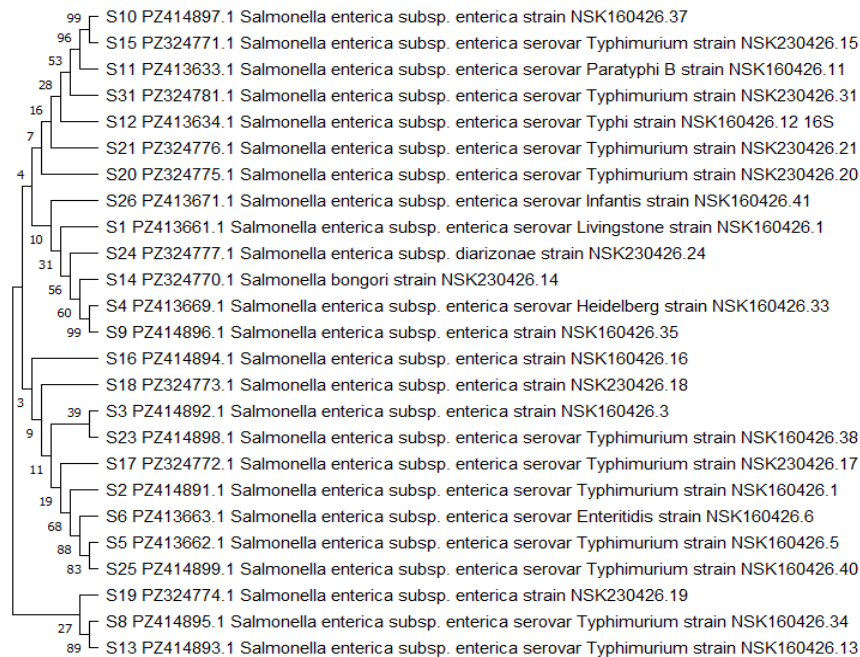
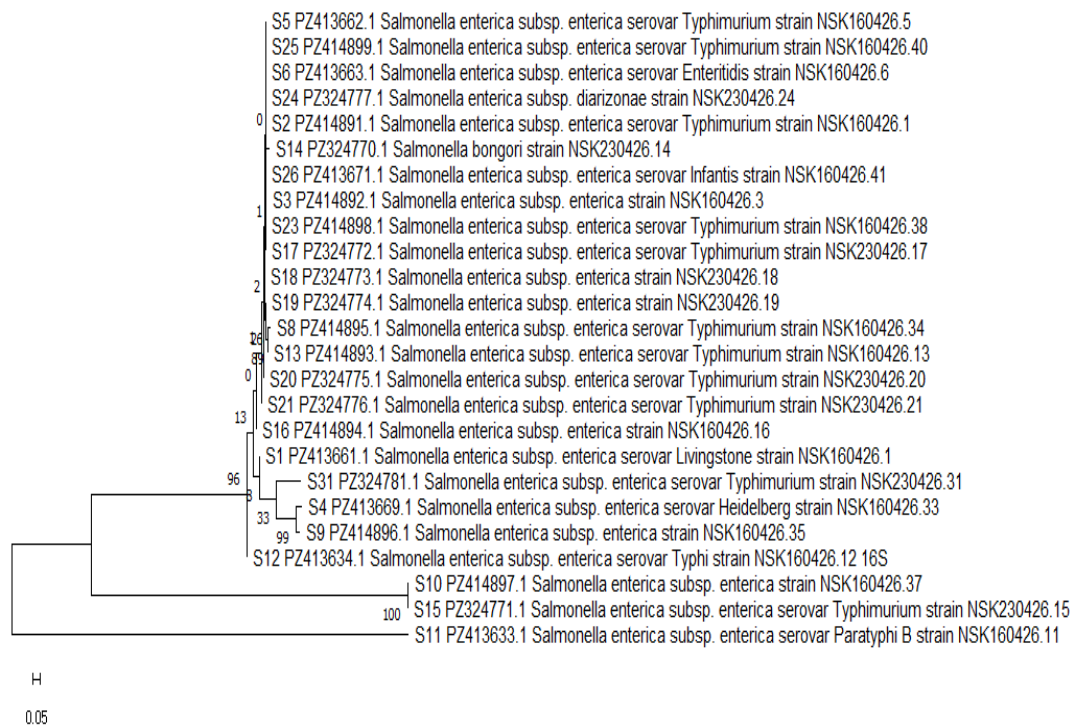
Table 6: Comparison of molecular identification and key biochemical characteristics

Sample ID	Molecular identification	Confirmed <i>Salmonella</i>	TSI reaction (Slant/Butt)	Non-lactose fermentation (K/A)	H ₂ S	Likely phenotypic interpretation
S1	<i>S. Livingstone</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S2	<i>S. Typhimurium</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S3	<i>S. enterica</i> (untyped)	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S4	<i>S. Heidelberg</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S5	<i>S. Typhimurium</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S6	<i>S. Enteritidis</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S7	<i>Citrobacter freundii</i>	No	K/A	Yes	+	High-risk misidentification
S8	<i>S. Typhimurium</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S9	<i>S. enterica</i> (untyped)	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S10	<i>S. enterica</i> (untyped)	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S11	<i>S. Paratyphi B</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S12	<i>S. Typhi</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S13	<i>S. Typhimurium</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S14	<i>S. bongori</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S15	<i>S. Typhimurium</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S16	<i>S. enterica</i> (untyped)	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S17	<i>S. Typhimurium</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S18	<i>S. enterica</i> (untyped)	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S19	<i>S. enterica</i> (untyped)	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S20	<i>S. Typhimurium</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S21	<i>S. Typhimurium</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S22	<i>Citrobacter youngae</i>	No	K/A	Yes	–	False presumptive <i>Salmonella</i>
S23	<i>S. Typhimurium</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S24	<i>S. diarizonae</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S25	<i>S. Typhimurium</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S26	<i>S. Infantis</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S27	<i>Klebsiella oxytoca</i>	No	K/A	Yes	+	High-risk misidentification
S28	<i>Enterobacter oligotrophicus</i>	No	K/A	Yes	–	False presumptive <i>Salmonella</i>
S29	<i>Enterobacter oligotrophicus</i>	No	K/A	Yes	–	False presumptive <i>Salmonella</i>
S30	<i>Enterobacter oligotrophicus</i>	No	K/A	Yes	–	False presumptive <i>Salmonella</i>
S31	<i>S. Typhimurium</i>	Yes	K/A	Yes	+	Typical <i>Salmonella</i>
S32	<i>Enterobacter hormaechei</i>	No	K/A	Yes	+	High-risk misidentification

Confirmed *Salmonella* = Molecularly identified as *Salmonella* spp.
 Non-*Salmonella* = Molecularly identified as other Enterobacteriaceae
 TSI pattern: K/A = non-lactose fermenter; A/A = lactose/sucrose fermenter
 H₂S: + = hydrogen sulfide production

Bootstrap values ranged from approximately 68% to 89% across the principal internal branches, while the basal lineage containing *S. Typhi*, *S. Paratyphi B*, and *S. Typhimurium* demonstrated very strong support (96–100%). These findings indicate that although the circulating isolates exhibited genetic diversity, they remained closely related evolutionarily and were clearly distinguishable from the non-*Salmonella* isolates identified by sequencing.

Overall, 16S rRNA sequencing reduced the estimated prevalence of *Salmonella* infection from 7.5% to 5.9% by eliminating seven false-positive phenotypic identifications. In addition to improving diagnostic accuracy, molecular characterization provided detailed information on species diversity and evolutionary relationships that could not be obtained using conventional laboratory methods alone.

Fig 5: Phylogenetic tree of the 25 molecular confirmed *Salmonella* isolates with bootstrapsFig 6: Phylogenetic tree of the 25 molecular confirmed *Salmonella* isolates without bootstraps

Discussion:

This study evaluated the diagnostic performance of conventional phenotypic methods relative to 16S rRNA gene sequencing for

the identification of *Salmonella* isolates recovered from blood cultures of patients with suspected enteric fever in Nigeria. The findings demonstrate that although routine biochemical methods remain useful as an initial screening

approach, they substantially overestimate *Salmonella* prevalence because of phenotypic similarities shared with other members of the family Enterobacteriaceae. Molecular confirmation using 16S rRNA sequencing significantly improved species identification, revealed important patterns of phenotypic misclassification, and provided phylogenetic evidence supporting the diversity of circulating *Salmonella* strains.

The blood culture positivity observed in this study is comparable to reports from other endemic regions of sub-Saharan Africa, where culture yields are frequently limited by prior antimicrobial exposure, delayed presentation to healthcare facilities, low-level bacteremia, and inadequate laboratory infrastructure (1-5). Although blood culture remains the reference standard for laboratory diagnosis of enteric fever, its sensitivity alone does not eliminate the possibility of species misidentification when isolate characterization relies exclusively on biochemical methods. Consequently, culture-positive isolates require accurate species confirmation before they are used for disease surveillance or antimicrobial resistance reporting.

One of the most important findings of this study was the discrepancy between phenotypic identification and molecular confirmation. Conventional biochemical testing initially classified all presumptive isolates as *Salmonella*; however, 16S rRNA gene sequencing demonstrated that a considerable proportion of these isolates were in fact members of other genera, including *Enterobacter*, *Citrobacter*, and *Klebsiella*. These findings are consistent with previous reports showing that several Enterobacteriaceae possess overlapping biochemical characteristics, including non-lactose fermentation, hydrogen sulphide production, and similar triple sugar iron (TSI) agar reactions, making differentiation by routine biochemical testing challenging (12,15-19).

The occurrence of false-positive *Salmonella* identification has important clinical implications. Patients may receive unnecessary or inappropriate antimicrobial therapy when treatment decisions are based solely on presumptive biochemical identification. Such inappropriate antimicrobial use contributes to selective pressure for antimicrobial resistance, increases healthcare costs, and exposes patients to avoidable adverse drug reactions. Furthermore, inaccurate identification compromises antimicrobial resistance surveillance by attributing resistance profiles to *Salmonella* when they may originate from other clinically significant Enterobacteriaceae (3,20,28,29).

The diagnostic performance analysis further demonstrated that conventional bioche-

mical methods possessed high sensitivity but comparatively lower specificity when evaluated against 16S rRNA sequencing. This finding indicates that while phenotypic screening is useful for minimizing missed *Salmonella* infections, it is insufficient as a definitive diagnostic method because of its susceptibility to false-positive results. The moderate agreement observed between phenotypic and molecular identification, as reflected by the Cohen's kappa statistic, reinforces the need for selective molecular confirmation, particularly for isolates intended for epidemiological surveillance, outbreak investigations, and antimicrobial resistance monitoring. Similar conclusions have been reported by recent studies evaluating molecular confirmation of foodborne pathogens in both clinical and public health laboratories (16-19,30).

Molecular sequencing identified several non-*Salmonella* organisms that had been incorrectly classified by biochemical methods. Species of *Enterobacter*, *Citrobacter*, and *Klebsiella* are well-recognized opportunistic pathogens capable of causing bloodstream infections, urinary tract infections, neonatal sepsis, and healthcare-associated infections. Their phenotypic resemblance to *Salmonella* has been documented previously and remains an important challenge in laboratories lacking access to molecular diagnostic technologies (15-19). Correct identification of these organisms is essential because they differ substantially from *Salmonella* in their epidemiology, virulence characteristics, and antimicrobial susceptibility patterns.

Phylogenetic analysis provided independent confirmation of the molecular identifications. The 16S rRNA gene sequences of confirmed isolates clustered closely with reference *Salmonella enterica* sequences, supporting the reliability of species assignment. The phylogenetic tree further demonstrated genetic diversity among circulating isolates through the presence of multiple well-supported clades, suggesting the circulation of several *Salmonella* lineages within the study population. Such genetic diversity has been increasingly reported in African surveillance studies and reflects the complex epidemiology of invasive salmonellosis in the region (3,9,14,31,32). Although 16S rRNA sequencing does not possess sufficient discriminatory power to differentiate closely related serovars or detect antimicrobial resistance genes, it remains a robust and cost-effective approach for confirming bacterial identity and validating routine microbiological diagnosis where whole-genome sequencing is not readily available (21-23). Furthermore,

even though antimicrobial susceptibility testing was not performed in this study, routine surveillance programmes should integrate standardized interpretive criteria, such as those provided by CLSI (14) and EUCAST (33), to ensure harmonized resistance monitoring across different settings.

From a public health perspective, the findings have important implications for surveillance systems in Nigeria and other resource-limited settings. Overestimation of *Salmonella* prevalence resulting from phenotypic misclassification may distort national disease burden estimates, bias antimicrobial resistance surveillance, and influence treatment guidelines and public health policy. Integrating molecular confirmation into routine laboratory workflows particularly within sentinel surveillance laboratories would substantially improve the quality of epidemiological data while supporting national antimicrobial resistance control programmes and the World Health Organization Global Antimicrobial Resistance and Use Surveillance System (GLASS) (20,29), as well as established programmes like the US CDC's National Antimicrobial Resistance Monitoring System (NARMS) (34).

The major strength of this study lies in the application of molecular sequencing to validate routine biochemical identification and characterize the phylogenetic relationships among clinical isolates. Unlike studies relying solely on conventional microbiology, the present investigation was able to identify false-positive *Salmonella* isolates and demonstrate the diversity of circulating strains. Nevertheless, several limitations should be acknowledged. The study was conducted within a limited geographical area, and the number of molecularly analysed isolates may not fully represent the diversity of *Salmonella* circulating nationally. In addition, identification based on 16S rRNA sequencing cannot reliably distinguish all *Salmonella* serovars or characterize antimicrobial resistance determinants.

While 16S rRNA gene sequencing offers superior diagnostic accuracy, its routine implementation in low-resource settings faces practical challenges, including high per-sample costs, infrastructure requirements, and technical expertise. However, a tiered diagnostic strategy where conventional biochemical methods serve as an initial screening tool, and molecular confirmation is reserved for surveillance activities, outbreak investigations, and isolates intended for antimicrobial resistance monitoring presents a pragmatic and cost-effective approach. This selective application minimizes costs while maximizing public health impact.

Compared to commercial automated identification systems such as API, Microbact, or VITEK, which are also cost-prohibitive for many laboratories in Nigeria, 16S rRNA sequencing offers the added advantage of providing phylogenetic information and enabling the detection of novel or emerging pathogens. As sequencing costs continue to decline and capacity-building initiatives expand across sub-Saharan Africa, molecular confirmation is becoming increasingly feasible for sentinel surveillance laboratories. Future studies employing whole-genome sequencing, multi-locus sequence typing, or core-genome phylogenetic analysis would provide higher-resolution epidemiological data to further strengthen these surveillance efforts.

Overall, the findings demonstrate that conventional biochemical methods remain valuable for initial laboratory screening but should not be relied upon as the sole basis for definitive identification of *Salmonella*. A tiered diagnostic strategy incorporating routine phenotypic screening followed by selective molecular confirmation would provide a practical and cost-effective approach for improving diagnostic accuracy, strengthening antimicrobial resistance surveillance, and enhancing enteric fever control programmes in endemic settings.

Conclusion:

This study demonstrates that conventional phenotypic methods, although useful for the initial screening of *Salmonella* isolates, are insufficient for definitive species identification because of substantial biochemical overlap with other members of the family Enterobacteriaceae. Molecular confirmation using 16S rRNA gene sequencing significantly improved diagnostic accuracy by distinguishing true *Salmonella* isolates from phenotypically similar organisms, thereby reducing the risk of misclassification.

The study further showed that reliance on biochemical identification alone can lead to overestimation of *Salmonella* prevalence, inaccurate antimicrobial resistance surveillance, and inappropriate clinical management. Phylogenetic analysis confirmed the taxonomic identity of the molecularly confirmed isolates and demonstrated genetic diversity among the circulating *Salmonella* strains, highlighting the value of molecular methods for epidemiological investigations.

The findings underscore the importance of incorporating molecular confirmation into routine laboratory workflows, particularly for surveillance activities, outbreak investigations,

and antimicrobial resistance monitoring. In resource-limited settings such as Nigeria, a tiered diagnostic approach combining conventional microbiological screening with selective 16S rRNA gene sequencing represents a practical and cost-effective strategy for improving diagnostic reliability and generating high-quality surveillance data.

Future studies should incorporate whole-genome sequencing and expanded multi-centre surveillance to provide higher-resolution characterization of *Salmonella* serovars, transmission dynamics, virulence factors, and antimicrobial resistance determinants. Such approaches will strengthen evidence-based public health interventions and contribute to improved control of enteric fever in endemic regions.

Contributions of authors:

CJA conceptualized the research, designed the methodology, performed the experiments, and wrote the original draft; CJA, DZE and PML analysed the data, curated the figures, and reviewed the manuscript; CJA provided resources, and DZE and PML supervised the project. All three authors read and approved the published version of the manuscript.

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Authors declare no conflict of interest

Artificial Intelligence (AI) use disclosure:

During the preparation of this work the authors used AI for editing purposes. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Data availability:

The molecular data in this study have been deposited in the NCBI GenBank database with the following accession numbers; PZ413661, PZ414891, PZ414892, PZ413669, PZ413662, PZ413663, PZ413664, PZ414895, PZ414896, PZ414897, PZ413633, PZ413634, PZ414893, PZ324770, PZ324771, PZ414894, PZ324772, PZ324773, PZ324774, PZ324775, PZ324776, PZ413665, PZ414898, PZ324777, PZ414899, PZ413671, PZ413667, PZ324778,

PZ324779, PZ324780, PZ324781, and PZ413668.

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