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Antimicrobial resistance profiling and molecular characterization of a multidrug-resistant *Salmonella enterica* serovar Typhimurium from poultry environments in Bangladesh

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ABSTRACT

The emergence of multidrug-resistant (MDR) *Salmonella enterica* in poultry production systems poses a significant threat to animal health, food safety, and public health worldwide. This study investigated the occurrence, antimicrobial resistance patterns, and molecular identity of zoonotic *Salmonella* spp. isolated from poultry environments in Bangladesh. A total of 60 samples, including cloacal swabs, tracheal swabs, fecal samples, and eggshell surfaces, were collected from poultry farms and analyzed using conventional culture-based methods and MALDI-TOF mass spectrometry. *Salmonella* spp. were detected in three samples, corresponding to an overall prevalence of 5%. Antimicrobial susceptibility testing of five *Salmonella* isolates, including three field isolates and two reference strains, was performed against 20 antibiotics representing multiple classes following CLSI guidelines. In contrast, most isolates exhibited broad susceptibility, one strain, designated *Salmonella* sp. ABD-261, displayed a multidrug-resistant phenotype, showing resistance to trimethoprim, gentamicin, tetracycline, and colistin sulfate. Molecular identification based on 16S rRNA gene sequencing and phylogenetic analysis confirmed ABD-261 as *Salmonella enterica* serovar Typhimurium. Notably, phylogenetic reconstruction revealed genetic divergence from closely related reference strains, suggesting potential evolutionary distinctiveness. The detection of an MDR zoonotic *S. enterica* serovar Typhimurium strain in poultry environments underscores the ongoing risk of antimicrobial resistance dissemination through the food chain. These findings highlight the importance of continuous surveillance, prudent antibiotic use, and strengthened biosecurity measures to mitigate the public health impact of poultry-associated *Salmonella* in Bangladesh.

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INTRODUCTION

Salmonella enterica serovar Typhimurium is a leading zoonotic pathogen responsible for significant global morbidity and economic losses, particularly through its association with foodborne outbreaks linked to poultry and poultry products (Majowicz *et al.*, 2010; Feasey *et al.*, 2012). In poultry production systems, *Salmonella* spp. can colonize the gastrointestinal and respiratory tracts of birds, contaminate farm environments, and persist through the food chain, posing a continuous threat to both animal and public health (EFSA, 2019). The emergence and spread of multidrug-resistant (MDR) *Salmonella* strains further complicate this scenario, limiting therapeutic options and increasing the risk of severe, untreatable infections in humans (Antunes *et al.*, 2016).

Bangladesh, with its rapidly growing and intensifying poultry sector, represents a critical hotspot for zoonotic disease transmission and antimicrobial resistance (AMR) selection (Rahman *et al.*, 2020). The widespread and often unregulated use of antibiotics in poultry farming for growth promotion, prophylaxis, and therapy has been identified as a key driver of AMR, fostering the selection of MDR bacterial pathogens, including *Salmonella* (Foysal *et al.*, 2024). Of particular concern is the development of resistance to critically important antimicrobials (CIAs), such as colistin, a last-resort antibiotic for treating infections caused by MDR Gram-negative bacteria (Liu *et al.*, 2016). The presence of MDR *Salmonella* in poultry environments thus constitutes a direct One Health challenge, bridging animal health, food safety, and human medicine (McEwen and Collignon, 2018).

Despite the recognized risks, surveillance data on the prevalence, serovar distribution, and resistance profiles of *Salmonella* from poultry environments in Bangladesh remain fragmented. Most studies have focused on clinical isolates or limited sample types, leaving significant gaps in understanding the ecology and transmission dynamics of MDR *Salmonella* within farm settings (Mahmud *et al.*, 2011). Comprehensive studies integrating conventional microbiology with molecular characterization are essential to map the resistance landscape, identify emerging strains, and trace their phylogenetic origins. In this context, the present study aimed to investigate the occurrence and antimicrobial resistance patterns of *Salmonella* spp. isolated from diverse poultry farm environments in Bangladesh. Furthermore, we sought to molecularly characterize a detected MDR isolate through 16S rRNA gene sequencing and phylogenetic analysis to elucidate its identity and evolutionary relationships. The findings underscore the imperative for sustained surveillance, prudent antimicrobial use, and enhanced biosecurity to mitigate the public health impact of poultry-associated MDR *Salmonella* in Bangladesh and similar settings worldwide.

MATERIALS AND METHODS

Isolation and pure culture establishment of *Salmonella* spp.

Poultry-associated samples, including tracheal swabs, cloacal swabs, eggshell swabs, and fecal samples, were aseptically collected from major retail poultry markets in the Ashulia and Savar areas of Dhaka, Bangladesh, using sterile cotton swabs pre-moistened with 4 mL of peptone buffer. The samples were immediately transferred into sterile, labeled tubes and transported to the laboratory under chilled conditions for processing. Upon arrival, each swab or fecal sample was suspended in 1.5 mL of sterile 0.2% magnesium chloride ($MgCl_2$) solution to facilitate bacterial release and homogenization. A 200 µL aliquot of each homogenate was subjected to tenfold serial dilution (10^{-1} to 10^{-5}) using sterile phosphate-buffered saline or peptone water.

From each dilution, 100 µL was spread-plated onto selective Salmonella-Shigella (SS) agar plates and incubated aerobically at 37°C for 24 hours. Colonies displaying typical *Salmonella* morphology, characterized by black centers due to hydrogen sulfide production, were presumptively selected and purified by repeated streaking on fresh SS agar plates to obtain single, well-isolated colonies. Clonal purity was confirmed by observing uniform colony morphology after subculturing on Luria-Bertani (LB)

agar. The purified isolates were propagated in LB broth at 37°C with shaking at 180 rpm and preserved in glycerol stocks at −80°C for downstream identification and further analyses.

Antibiotic susceptibility test

The antimicrobial susceptibility of the *Salmonella* isolates was evaluated using the standardized disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines, with minor modifications as described by Thai *et al.* (2012).

Fresh bacterial cultures were uniformly inoculated onto the surface of Salmonella-Shigella (SS) agar plates using sterile cotton swabs to achieve a confluent lawn, ensuring even distribution by streaking in multiple directions, followed by air-drying for 5 minutes.

Commercial antibiotic disks (Himedia, USA) were aseptically placed on the agar at a distance of 24 mm and gently pressed to ensure optimal contact. Plates were incubated at 37°C for 24 hours in an inverted orientation, and the diameters of inhibition zones were measured in millimeters. Susceptibility was assessed against a panel of 20 antibiotics, including ampicillin (10 µg), amikacin (30 µg), gentamicin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), ceftazidime (30 µg), imipenem (10 µg), azithromycin (30 µg), ciprofloxacin (5 µg), fosfomycin (50 µg), colistin (10 µg), nitrofurantoin (300 µg), netilmicin (30 µg), cefotaxime (30 µg), trimethoprim (10 µg), tigecycline (15 µg), levofloxacin (5 µg), cefixime (5 µg), doripenem (10 µg), and cefuroxime (30 µg). Zone diameters were interpreted as susceptible (S), intermediate (I), or resistant (R) according to CLSI breakpoints (CLSI, 2020). Isolates demonstrating non-susceptibility to at least one agent in three or more antimicrobial classes were classified as multidrug-resistant (MDR), following the criteria proposed by Magiorakos *et al.* (2012).

Identification of isolated strains

Bacterial isolates were preliminarily identified at the species level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Corporation, USA). Fresh colonies were applied to the target plate, overlaid with matrix solution, and analyzed against a validated reference database. High-confidence identification scores enabled provisional assignment at the genus and species level. Genomic DNA was extracted from purified isolates using a commercial DNA extraction kit, and DNA purity and concentration were assessed spectrophotometrically (260/280 nm) to ensure suitability for downstream molecular applications. Species-level confirmation was performed via PCR amplification of conserved Salmonella-specific genes and the universal 16S rRNA gene, with amplicons resolved on 1.5% agarose gels and visualized under UV illumination. Representative 16S rRNA amplicons were purified and subjected to Sanger sequencing, and the resulting sequences were compared against the NCBI GenBank database using BLAST.

Phylogenetic relationships were inferred using MEGA software to validate species-level classification. This integrated proteomic and molecular approach provided definitive species identification, ensured clonal purity, and facilitated correlation of genetic identity with phenotypic traits, including antimicrobial resistance, enabling comprehensive characterization for epidemiological and diagnostic analyses.

Phylogenomic relatedness among *Salmonella* isolates

To elucidate the evolutionary relationships of the *Salmonella* isolates, 16S rRNA gene sequences were aligned using the ClustalW algorithm implemented in MEGA X software.

Phylogenetic trees were reconstructed employing the Maximum Likelihood (ML) method, and the statistical support for each branch was evaluated using 1,000 bootstrap replicates.

Evolutionary distances were computed according to the Kimura two-parameter model, while gaps and missing positions were handled by pairwise deletion to maximize data integrity.

Reference *Salmonella* sequences retrieved from the NCBI GenBank database were included to contextualize the isolates within established phylogenetic lineages. The resulting phylogenetic topology provided robust insights into the genetic relatedness and species-level classification of the isolates, facilitating molecular epidemiological comparisons and supporting the correlation of genotypic traits with phenotypic characteristics, including antimicrobial resistance patterns.

RESULTS

Isolation of *Salmonella* spp. from poultry and poultry farming environments

A total of 60 samples were collected from poultry-associated sources, including cloacal swabs, tracheal swabs, fecal samples, and eggshell surfaces, as presented in Table 1. Among these, three samples were confirmed positive for *Salmonella* spp., corresponding to an overall prevalence of 5%. The positive samples comprised one cloacal swab, one tracheal swab, and one fecal sample, each representing a prevalence of 6.67% within their respective categories (Table 1).

Table 1. Sample types and distribution of positive *Salmonella* isolates

Sample type	Number of samples	Positive sample	Prevalence (%)
Cloacal swab	15	01	6.67%
Tracheal swab	15	01	6.67%
Feces	15	01	6.67%
Eggshell surface	15	00	0%
Total	60	03	5%

Initial identification was based on conventional culture and colony morphology, followed by confirmation using Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry, ensuring accurate genus-level identification. On Salmonella-Shigella (SS) agar, presumptive *Salmonella* colonies exhibited characteristic morphology, appearing as colorless to translucent colonies with black centers due to hydrogen sulfide (H_2S) production (Fig. 1). The distribution of positive isolates across sample types is summarized in Table 1.

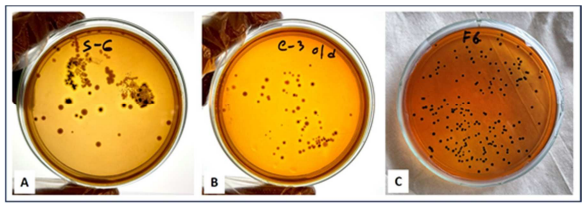

Fig. 1. Colony morphology of isolated *Salmonella* spp. A) *Salmonella* sp. ABD-245; B) *Salmonella* sp. ABD-255; C) *Salmonella* sp. ABD-261

These findings indicate that *Salmonella* spp. is present in key biological niches within poultry production systems, particularly in the gastrointestinal and respiratory tracts of birds. Although the overall prevalence was relatively low, the detection of the pathogen in both cloacal and fecal samples highlights its potential for dissemination through excretory routes.

The presence of *Salmonella* in these sources underscores the risk of environmental contamination and subsequent zoonotic transmission. Therefore, continuous surveillance, improved farm hygiene, and strengthened biosecurity measures are essential to minimize the public health risks associated with poultry-borne *Salmonella*.

Antibiotic sensitivity profile of isolated *Salmonella* spp. strains

A total of five *Salmonella* isolates comprising three native wild-type strains and two ATCC reference strains, all identified as serovar Typhimurium (Table 2), were subjected to antimicrobial susceptibility testing against a panel of 20 antibiotics from various classes. The antibiotic susceptibility of the isolated *Salmonella* strains was evaluated using the Kirby-Bauer disk diffusion method, following the Clinical and Laboratory Standards Institute (CLSI) guidelines. Zones of inhibition were measured, and isolates were classified as Sensitive (S), Intermediate (I), or Resistant (R) based on standard interpretive criteria. The majority of the isolates exhibited broad-spectrum susceptibility, indicating low levels of antimicrobial resistance within the tested group.

Table 2. Antibiotic resistance assays of *Salmonella* strains

Sl	Antibiotics name	Antibiotic content per disc (µg)	Bacterial strains				
			ATCC13311	ATCC 14028	ABD-245	ABD-255	ABD-261
01	Nitrofurantoin	F- 300	S	S	I	S	I
02	Netilmicin	NET- 30	S	I	S	S	I
03	Ceftazidime	CAZ- 30	S	S	I	S	S
04	Cefotaxime	CTX- 30	S	S	S	S	S
05	Trimethoprim	TR- 10	S	S	S	S	R
06	Gentamicin	GEN- 10	S	I	I	S	R
07	Tigecycline	TGC- 15	S	S	S	S	S
08	Levofloxacin	LVX- 5	S	S	S	S	S
09	Ciprofloxacin	CIP- 5	S	S	S	S	S
10	Cefixime	CFM- 5	S	S	S	S	S
11	Azithromycin	AZM- 30	S	S	S	S	S
12	Imipenem	IMP- 10	S	S	S	S	S
13	Cefuroxime	CXM- 30	S	I	S	S	S
14	Ampicillin	AMP- 10	S	I	S	S	S
15	Chloramphenicol	C- 30	S	S	S	S	S
16	Amikacin	AK- 30	S	S	S	S	S
17	Tetracycline	TE- 30	S	S	S	S	R
18	Colistin Sulphate	CL- 10	I	I	I	S	R
19	Doripenem	DOR- 10	S	S	S	S	S
20	Fosfomycin	FO- 50	S	S	S	S	I

Notes: Sensitive (S), Intermediate (I), or Resistant (R)

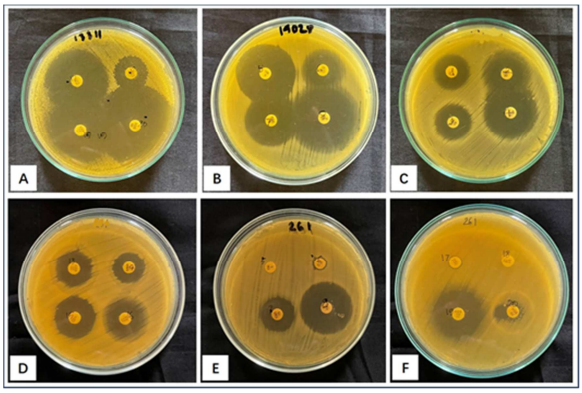

Fig. 2. Antibiotic sensitivity assays of *Salmonella* spp. strains. A) ATCC 13311; B) ATCC 14028; C) ABD-245; D) ABD-255; E-F) ABD-261

However, one strain, designated *Salmonella sp*. ABD-261, demonstrated notable multidrug (MDR) resistance (Table 2; Fig. 2). This strain was resistant to four different classes of antibiotics: Trimethoprim (a folate pathway inhibitor), Gentamicin (an aminoglycoside), Tetracycline (a protein synthesis inhibitor), and Colistin Sulfate (a polymyxin targeting the bacterial outer membrane). This multidrug-resistant phenotype raises significant concern regarding potential treatment challenges and highlights the importance of ongoing antimicrobial resistance surveillance in poultry-associated *Salmonella* strains.

Molecular characterization of MDR *Salmonella sp*. strain ABD-261 based on 16S rRNA gene analysis

To investigate the genetic identity and evolutionary positioning of multidrug-resistant (MDR) *Salmonella sp*. strain ABD-261, the 16S rRNA gene was PCR-amplified (Fig. 3) and sequenced. A phylogenetic tree was constructed using the Maximum Likelihood (ML) method, incorporating 88 representative 16S rRNA sequences from the genera *Salmonella* and related members of the Enterobacteriaceae family (Fig. 4).

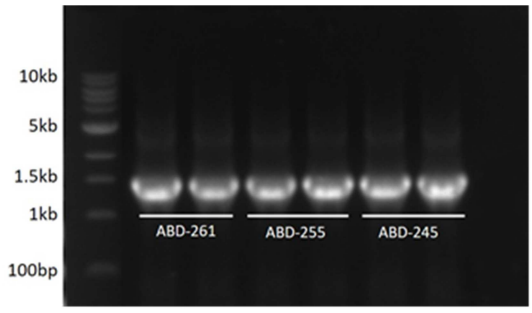

Fig. 3. Amplification of the 16S rRNA gene in *Salmonella* spp.

The resulting phylogeny delineated four distinct clades, with strain ABD-261 clustering exclusively within the *Salmonella* genus.

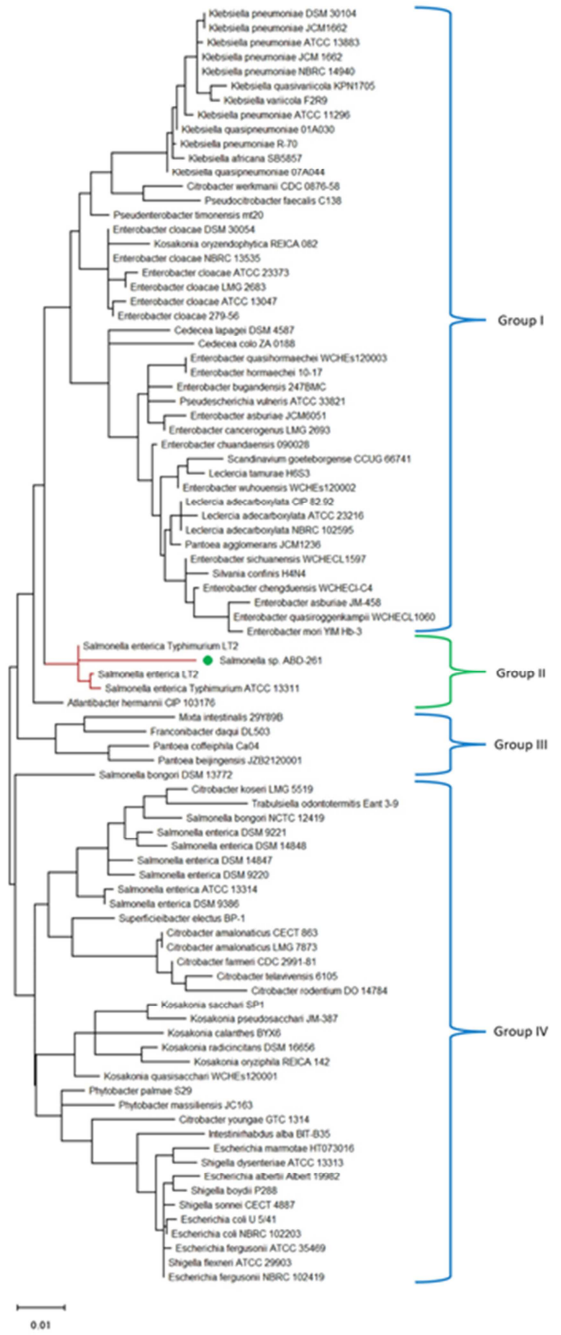

Fig. 4. Phylogenetic tree based on the 16S rRNA gene sequences of *Salmonella* and related bacterial species The studied MDR strain *Salmonella* spp. ABD-261 is shown in a green circle, and the scale bar specifies 0.01 expected changes per site.

Notably, ABD-261 formed a well-supported and discrete node, closely allied with *Salmonella enterica* serovar Typhimurium LT2 and *S. enterica* Typhimurium ATCC 13311. The strong bootstrap support further validated the phylogenetic placement of ABD-261, indicating a close evolutionary relationship with these reference strains. This clustering pattern is consistent with the morphological and biochemical traits observed in strain ABD-261, characteristic of *S. enterica* serovar Typhimurium. Furthermore, the ML dendrogram revealed significant genetic divergence between ABD-261 and its closest relatives, suggesting potential genetic novelty.

DISCUSSION

Prevalence and context of *Salmonella* in poultry environments

The present study provides critical insights into the occurrence, antimicrobial resistance profiles, and molecular identity of *Salmonella enterica* serovar Typhimurium isolated from poultry farm environments in Bangladesh. The detection of *Salmonella* spp. in cloacal swabs, tracheal swabs, and fecal samples, albeit at a relatively low prevalence (5%), underscores the persistent colonization of poultry by this pathogen. This finding aligns with previous reports from Bangladesh and other regions, where *Salmonella* has been frequently isolated from internal bird samples, highlighting the gastrointestinal and respiratory tracts as key reservoirs (Mahmud *et al.*, 2011; Popy *et al.*, 2025). The absence of *Salmonella* on eggshell surfaces in our study may be attributed to effective farm-level hygiene practices or sampling limitations.

However, this result contrasts with studies reporting significant contamination of eggs in similar settings (Gast *et al.*, 2019). The low overall prevalence observed could reflect variations in farm management, biosecurity levels, or regional epidemiological patterns, emphasizing the need for context-specific surveillance (Cardinale *et al.*, 2004).

Antimicrobial resistance and molecular characterization of a multidrug-resistant *Salmonella enterica* serovar Typhimurium isolate

The antimicrobial susceptibility testing revealed a concerning multidrug-resistant phenotype in one isolate, *Salmonella* sp. ABD-261, which exhibited resistance to trimethoprim, gentamicin, tetracycline, and colistin sulfate.

The resistance to colistin, a polymyxin antibiotic of last resort for treating infections caused by carbapenem-resistant Enterobacteriaceae, is particularly alarming (Liu *et al.*, 2016). The emergence of colistin-resistant *Salmonella* in poultry environments has been increasingly reported in Asia, often linked to the widespread use of colistin in animal production and the presence of mobile genetic elements such as the *mcr* genes (Wang *et al.*, 2017; Elbediwi *et al.*, 2019). While this study did not investigate the genetic determinants of resistance, the observed MDR pattern suggests the potential accumulation of resistance genes, possibly facilitated by the selective pressure from indiscriminate antibiotic use in Bangladesh's poultry sector (Foysal *et al.*, 2024). The susceptibility of other isolates to most antibiotics tested is encouraging but should be interpreted with caution, given the small sample size and the possibility of undetected resistance genes.

Molecular identification based on 16S rRNA gene sequencing and phylogenetic analysis unequivocally classified the MDR strain ABD-261 as *Salmonella enterica* serovar Typhimurium, clustering closely with reference strains LT2 and ATCC 13311. This serovar is a predominant cause of non-typhoidal salmonellosis globally and is frequently associated with poultry-borne outbreaks (EFSA, 2019). Notably, the phylogenetic reconstruction revealed significant genetic divergence between ABD-261 and its closest relatives, suggesting potential evolutionary distinctiveness. Such divergence may indicate local adaptation, the acquisition of foreign genetic material (e.g., plasmids harboring resistance genes), or the emergence of a novel lineage within the region (Zhou *et al.*, 2020). While 16S rRNA gene analysis provides reliable genus and species identification, further genomic characterization using whole-genome sequencing would be invaluable to elucidate the specific resistance mechanisms, virulence factors, and phylogenetic relationships at a higher resolution (Deng *et al.*, 2016).

The detection of an MDR *S.* Typhimurium strain in a poultry farm environment carries significant One Health implications. Poultry serves as a critical link in the transmission of resistant bacteria to humans through direct contact, environmental contamination, or the food chain (McEwen and Collignon, 2018). The resistance profile of ABD-261, including last-resort colistin, poses a direct threat to public health by narrowing therapeutic options for severe salmonellosis. This scenario is particularly concerning in Bangladesh, where antibiotic stewardship in both veterinary and human medicine remains a challenge, and surveillance systems for AMR in food-producing animals are still developing (Rahman *et al.*, 2020).

In conclusion, this study highlights the presence of a multidrug-resistant *Salmonella enterica* serovar Typhimurium strain in Bangladesh's poultry environment, signaling a potential reservoir for antimicrobial resistance transmission. These findings reinforce the urgent need for integrated surveillance programs that monitor AMR trends in zoonotic pathogens across the farm-to-fork continuum. Future research should focus on larger-scale epidemiological studies, genomic analysis of resistant isolates, and investigation of the drivers of resistance selection in poultry farming systems. Strengthening biosecurity measures, promoting the prudent use of antibiotics, and implementing One Health interventions are essential steps to mitigate the public health impact of MDR *Salmonella* in Bangladesh and similar settings worldwide.

AUTHORSHIP

The first and second authors contributed equally to this study.

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