

Genomic Insights into Multidrug Resistance, Virulence, and Phage Defense in Poultry-Derived *Shigella sonnei* from Bangladesh

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Abstract

The emergence and dissemination of antimicrobial resistance (AMR) in zoonotic pathogens represent a pressing global health challenge, particularly in low- and middle-income countries where poultry farming plays a key socio-economic role. In this study, we employed next-generation sequencing and comprehensive bioinformatics pipelines to characterize a novel bacterium *Shigella sonnei* BCSIR-MHS-F2 strain, isolated from a poultry rectal swab in Bangladesh, the first such report from the country. This strain is moderately resistant against gentamicin, tazobactam, and strongly resistant against ampicillin and ceftriaxone. According to crystal-violet assay, this bacterium is a moderate biofilm former. The genome assembly yielded 167 contigs, resulting in a genome length of 5,045,727 base pairs (bp) with a G + C content of 50.58%. Key metrics, such as a contig N50 of 134,475 bp and a contig L50 of 11 underscore the quality of the assembly, providing a solid foundation for further genomic analysis. Genome-wide analysis revealed the presence of multiple antimicrobial resistance genes (ARGs), including *bla*TEM, *bla*CTX-M (β -lactam), *tetA/B* (tetracycline), *sul1* (sulfonamide), *qnrS* (fluoroquinolone), and *ermB* (macrolide), frequently associated with mobile genetic elements such as plasmids, integrons, and transposons, indicating a high potential for horizontal gene transfer. This bacterium contains the Type III Secretion System, which plays a big role in virulence. By going through evolutionary trees and studying their antibiotic resistance trends, we could unveil how this strain evolved mechanism and how antibiotic resistance spreads. This is a clear wake-up call that we urgently need to monitor antibiotic resistance where humans, animals, and the environment meet. By taking a 'One Health' approach, our research provides the data needed to improve antibiotic use and farm safety across Bangladesh's poultry industry and beyond.

Introduction

Shigella sonnei is a bacterial pathogen that is predominantly classified as a cause of human gastrointestinal infections. However, its presence in poultry environments has raised concerns regarding public health and food safety. This strain is a nonmotile, non-spore-forming, gram-negative rod-shaped bacteria. Alongside, *Shigella flexneri*, another bacterium, which is accountable for approximately 90% of shigellosis (bacillary dysentery) cases worldwide¹. However, *S. sonnei* is particularly noteworthy for its escalating prevalence in both developed and developing countries, frequently replacing *S. flexneri* as the primary cause of shigellosis in regions with increased sanitation^{1,2}. The genomic data indicates that *S. sonnei* has recently undergone a rapid clonal expansion from its European origin, resulting in the emergence of epidemic lineages that are currently circulating worldwide. The dissemination of *S. sonnei* clones has been facilitated by modern travel and commerce, resulting in outbreaks in regions where it was previously uncommon^{3,4}. The prevalence is also associated with retardation of cross-immunity from other related pathogenic bacteria.

This strain is highly present in poultry-related samples, like eggshells, water, and feed. According to a study, in ten-layer farms, *S. sonnei* was prevalent in 14% feeds, 11% water and 6% eggshells. The findings suggests that poultry environments can be a habitat for *S. sonnei*⁵. This strain is frequently transmitted from the fecal-oral route, person-to-person contact, and contaminated food or water (CDC, WHO). A few 10 to 100 *S. sonnei* is enough to make one destabilizing disease form^{2,6}. Even though symptoms are being resolved, infected humans can excrete bacteria in their stools for weeks.

The most common symptoms are watery or bloody diarrhea, abdominal pain, cramping, fever, nausea, vomiting, loss of appetite, headache, and malaise. The majority of infections are self-limiting, with a resolution time of approximately one week. However, in vulnerable populations, severe or protracted illness may occur. The intestinal mucosa is invaded by *S. sonnei*, which then polymerizes the actin of the host cell to facilitate cell-to-cell transmission. It is equipped with a variety of virulence mechanisms, such as the production of mucinases (enzymes that degrade mucins), colicins (toxins that destroy related bacteria), and proteins that disrupt intestinal epithelial integrity ².

Recent research has identified *Shigella* strains that can produce Shiga toxin through the acquisition of a prophage, which has raised concerns about the potential for virulence to evolve. There is a growing correlation between *S. sonnei* and extensively drug-resistant (XDR) and multidrug-resistant (MDR) infections, which include resistance to azithromycin and ciprofloxacin ¹. Because this bacterium resists antibiotics, treating infections is becoming much harder, creating major public health risks especially when large outbreaks hit. Interestingly, as communities improve their sanitation and overall living standards, we are seeing a shift in the type of bacteria causing these illnesses.

Shigella sonnei is becoming the most common etiological agents of Shigellosis and outpacing other species like *S. flexneri*, *S. dysenteriae*, and *S. boydii*. The multifactor reason is working behind for this shifting. The acquisition of natural cross-immunity from *Plesiomonas shigelloides*, a bacterium that shares an O-antigen with *S. sonnei*, in poor water sanitation, giving an upper hand. When water supplies improve, exposure and natural environmental immunity may decline and later, populations are increasingly vulnerable to *S. sonnei* ^{4,7}. As hygiene improved and sanitation, the total bacterial exposure is limited and hence, *S. sonnei* used to dominate over other species ^{3,7}. According to study, as nations industrialize and socio-economic status rises, *S. sonnei* becoming prevalent and causes Shigellosis ^{7,8}. China, Vietnam, Bangladesh, and other industrializing regions of Asia and Latin America have witnessed this shift ^{3,7}. This outpacing shift is also linked to better nutritional status, which is basically the interaction between the host exposures and immunological response ⁸. The rampant use of antibiotics is contributing to multi-drug resistant patterns of bacterial species ⁹. *S. sonnei*'s is able to pick up antimicrobial resistance genes from horizontal gene transfer events and its give them a selective advantage in areas with high or uncontrolled antibiotic use ⁹.

The whole genome sequencing (WGS) is a thorough examination of the organism's genetic composition ¹⁰. This technique has been extensively adopted in life sciences as a result of recent advancements ¹¹. By going through bacterial genome insights, it is possible to unveil antimicrobial resistance, virulence factors, mobile genetic elements, CRISPR-cas systems, and metabolite gene clusters ¹²⁻¹⁴. Later, those information provides the possible therapeutic mechanism to contain this bacterium and compare with existing therapies ^{15,16}. The successful tactics to tackle any dangerous bacteria depends on the wealth of information ¹⁷. The outpacing shift of *S. sonnei* needs continuous surveillance, research into vaccine development, and strategies to combat antimicrobial resistance.

As a potential threat to poultry infections in Bangladesh, *Shigella sonnei* has not been extensively reported. Nevertheless, it is important to consider its potential contribution to these infections. There is currently no published whole genome sequencing data for *Shigella sonnei* from Bangladeshi poultry. The concern and importance of investigating this infectious pathogen in Bangladesh were prompted by the heedless inattention

to it. We have reported the first complete genome sequence of *Shigella sonnei* from poultry in Bangladesh (nosocomial orientated *Shigella sonnei* has already occurred). In addition to identifying the isolate's biofilm-forming capacity and antibiotic susceptibility pattern, we have analyzed the entire genome sequence to evaluate its virulence genes, antimicrobial resistance genes and mechanisms, mobile genetic elements, biosynthetic gene clusters, subsystems, and metabolic pathways. Furthermore, we have conducted comparative genomics and pathogenicity profiling on the genomes of *Shigella sonnei* and *Escherichia spp* from human and non-human sources that have been published worldwide. This has allowed us to gain insight into the genomic diversity of the organism and to make predictions regarding the cross-species transmission of the pathogen.

Results

Isolation, Genome Assembly and Phylogenetic Analysis of *Shigella sonnei* BCSIR-MHS-F2

Rectal swabs were obtained from chicken, enriched on nutrient broth in incubator for 24 hours at 37°C. After turbidity indicated the enriched bacterial broth, 100 micro-L broth was spread out onto XLD plates and incubated at 37°C for 18 to 24 hours. A single well-isolated colony was picked and sub-cultured on a fresh XLD plate using the quadrant streak method. After again 24 hours of incubation at 37°C, pure, well-separated pink colonies without black centers confirmed successful subculture and purity of the presumptive *Shigella* isolate (Fig. 1a)

Genomic DNA isolate was extracted using the QIAamp® DNA Mini kit according to the manufacturer's instructions. DNA was eluted in AE buffer and preserved it at -22°C. The quality and quantity of the extracted DNA were assessed by using Nanodrop and confirmed by gel electrophoresis to happen the required standards for sequencing. The extracted DNA concentration was 39.6 ng/ul and the purity is 1.85. After MiniSeq sequencing platform, the FASTQ file was streamed into BV-BRC platform for genome assembly.

The genome assembly for presumptive *Shigella* BCSIR-MHS-F2 was performed using the Comprehensive Genome Analysis service at BV-BRC (Fig. 1b). The assembly produced 167 contigs with a G + C content of 50.58% and a genomic length of 5,045,727 base pairs (bp). Plasmids were not found in the assembly. Important metrics that highlight the assembly's quality and provide a strong basis for additional genomic study include a contig N50 of 134,475 bp and a contig L50 of 11 (Table 1).

As shown in Fig. 1c, *Shigella sonnei* ATCC 29930 and *Shigella boydii* ATCC 8700 are the two species that the BCSIR-MHS-F2 isolate most closely aligns. BCSIR-MHS-F2 isolate also belongs to a monophyletic group (bootstrap support > 90%) with *S. flexneri* and *S. dysenteriae*, which demonstrates considerable genomic similarity. The phylogenetic position of BCSIR-MHS-F2 within *Shigella* lineage and distance away from pathogenic *Escherichia coli* and non-pathogenic *Escherichia species* (*E. marmotae*, *E. ruysiae*, and *E. fergusonii*). The tress also shows that the isolate is clearly different from basal outgroups like *Escherichia hermannii* and *Escherichia albertii*, and its genome is more closely related to enteroinvasive strains.

Table 1
Assembly Details.

Feature	Value
Contigs	167
GC Content	50.58%
Contig L50	11
Genome Length	5,045,727 bp
Contig N50	134,475

Identification of *Shigella sonnei* Lineage

Average Nucleotide Identity (ANI) was calculated utilizing the Integrated Prokaryote Genome and Pan-genome study (IPGA) platform (Fig. 2a). It is a genomic comparison on nucleotide-level among microbial genomes. If the ANI score is greater than 95–96%, this indicates the species-level relatedness. According to Fig. 2a, *Shigella* BCSIR-MHS-F2 has high ANI with *Shigella boydii* and *Shigella sonnei*. BCSIR-MHS-F2 belongs to the *Shigella* species group which indicates high genetic similarity and a common evolutionary origin. *Shigella flexneri* and *Escherichia coli* K-12 also exhibited high ANI values with BCSIR-MHS-F2, which establishes their genomic proximity. On the other side, *Escherichia fergusonii* and *Escherichia albertii* showed lower ANI values and cluster apart, which indicates their genomic divergence. In those investigations, *Shigella sonnei* BCSIR-MHS-F2's taxonomic position is strongly supported and validates the strong link to other clinically significant *Shigella* and *E. coli* isolates.

Shigella BCSIR-MHS-F2 has significant genetic overlap and good conservation of core genomic material when compared to other *Shigella sonnei*, *Shigella boydii*, *Shigella flexneri*, and *Escherichia coli* K-12 in pan-genome study (Fig. 2b). The evolutionary links between *Shigella* species and *E. coli* are supported by this study. The image illustrates how the core genome shows a declining trend when additional genomes are included in the study, indicating that fewer genes are shared by all strains of *Shigella*. Essential housekeeping genes are conserved in bacterial species, although strain-specific accessory genes differ. The *Shigella* species appears to have a reasonably stable conserved genetic backbone, as seen by the core genome appearing to plateau at 2,500 gene clusters. The pan-genome, on the other hand, surpasses 11,000 gene clusters in the subgroup and keeps growing with every new genome (Fig. 2c). The result suggests that *Shigella* is an open pangenome with great genetic variation and ability to adapt in different host or environmental conditions. Hence, it is also justifying that *Shigella* species can acquire virulence, antibiotic resistance, or niche-specific metabolism by horizontal gene transfer events.

According to Fig. 2b, *Shigella* BCSIR-MHS-F2 shared many gene clusters with *Escherichia coli* K-12, *S. sonnei*, *S. flexneri*, *S. boydii*, and *S. dysenteriae*, which highlights a strong link between *E. coli* and *Shigella*. On the contrary, *Shigella* BCSIR-MHS-F2 have more distinct gene clusters with *Escherichia whittamii*, *E. marmotae*, *E. ruysiae*, which emphasizes the divergent genome (Fig. 2d). These distinct genes most likely reflect strain-specific adaptations or characteristics linked to a particular environment. The pan-genome organization of *Shigella* and *E. coli*, where the accessory genome is diverse and vast and the core genome is comparatively tiny and

conserved, is well depicted by this junction plot. This variety highlights the genetic variability within this bacterial group and promotes the strains ecological adaptation and versatility.

Genome Annotation and Functional Insights

After genome assembly, the FASTA sequence was retrieved and submitted to PubMLST database for species identification. The findings revealed that the bacterium is *Shigella sonnei*. Using the RAST tool kit (RASTtk) in PATRIC, the *Shigella* BCSIR-MHS-F2 genome was annotated, yielding 5,106 protein-coding sequences (CDS), 81 transfer RNA (tRNA) genes, and 3 ribosomal RNA (rRNA) genes. This genome annotation provides coding potential and a set of structural RNA components for cellular function and protein synthesis respectively. The *Shigella* genome consists of 4,604 proteins with functional assignments, 502 hypothetical proteins, 906 proteins related to KEGG pathways, 1,314 relates to Enzyme Commission (EC) numbers, and 1,083 are linked to Gene Ontology (GO). The functional variety of the *Shigella* genome is further demonstrated by the presence of 5,001 proteins assigned to cross-genus protein families (PGFam) and 4,932 proteins allocated to genus-specific families (PLFam) (Table 2).

Table 2
Annotated Genome Features.

Feature	Value
CDS	5,106
tRNA	81
rRNA	3
Repeat Regions	25
Hypothetical proteins	502
Proteins with functional assignments	4,604
Proteins with EC number assignments	1,314
Proteins with GO assignments	1,083
Proteins with Pathway assignments	906
Proteins with PATRIC genus-specific family (PLfam) assignments	4,932
Proteins with PATRIC cross-genus family (PGfam) assignments	5,001

The *Shigella* BCSIR-MHS-F2 genome was rapidly annotated using subsystem technology (RAST), and categorized the predicted genes into functional subsystems ¹⁸. The findings revealed that metabolism is the largest category, consisting of 116 functional subsystems and 952 genes. This defines the *Shigella's* strong metabolic function, which is utmost important for biosynthesis, energy generation, and nutrient processing ^{19,20}. In protein processing category, 268 genes are spread across 46 subsystems, which is related to protein folding, modification, degradation and cellular homeostasis. Stress response, defense, and virulence consist of 208 genes and 45 subsystems and highlights the *Shigella's* ability to interact with hosts, survive in harsh environments, and pathogenicity. *Shigella's* metabolic energy generation is reflected in the Energy category

which consists of 336 genes and 36 subsystems. Other functional categories include membrane transport (169 genes), cellular processes (178 genes), DNA processing (115 genes), and RNA processing (75 genes) indicate cellular integrity, replication and gene expression of *Shigella* (Fig. 3a). Structural stability and regulation mechanisms are controlled by cell envelopes, miscellaneous activities, and regulation and cell signaling. Overall, this subsystem distribution shows a potentially pathogenic, metabolically active, and environmentally tolerant genome, which is consistent with traits seen in other clinically relevant *Shigella* strains.

The Clusters of Orthologous Groups (COG) (Fig. 3b) and KEGG (Fig. 3c) annotations indicate that the genome of *Shigella sonnei* BCSIR-MHS-F2 is predominantly enriched in genes associated with metabolism, followed by functions involved in information storage and processing, environmental adaptation, and cellular processes. The abundance of genes involved in carbohydrate metabolism, amino acid metabolism, membrane transport, transcription, translation, and DNA repair reflects the bacterium's ability to efficiently exploit host nutrients, maintain genome integrity, and adapt to environmental stress.

Antimicrobial Resistance Profiling and Mapping of the Virulence Island and Virulence Factor Genes of *Shigella sonnei* BCSIR-MHS-F2

Shigella BCSIR-MHS-F2 exhibits a number of antimicrobial resistance gene pools. Significantly, a high percentage of genes, including *TEM-1*, *sul2*, *mdtB*, and *qnrS1*, show that they are widely and consistently present in the poultry microbiome. Efflux pumps (*mdtK*, *acrF*) and β -lactamases (*blaEC*, *ACT-5*, *OXA*), many of which are known to contribute to multidrug resistance, were among the strain's notable variety. These resistance genes are linked to host species by the outer ring (Fig. 4a), which is dominated by *Escherichia coli* and *Klebsiella pneumoniae*, two important sources of transferable resistance genes in human and poultry diseases.

The Table 3 highlights the preponderance of well-characterized threats and predicts homologous resistance genes. In Bangladesh's food supply chain, poultry, play a crucial role in harboring and spreading clinically relevant AMR genes that may endanger human and veterinary health.

Table 3

Antimicrobial Resistance Genes of *Shigella* BCSIR-MHS-F2. The Antibiotic Resistance Ontology (ARO) categorization of resistance genes found in *Shigella* BCSIR-MHS-F2, as determined by the CARD online server. The RGI Criteria classifies AMR genes as "Perfect, Strict, and Loose" based on prediction confidence.

Mechanism	Gene	RGI Criteria	AMR Gene Family	Drug Class	% Identity	% Length
Antibiotic Efflux	<i>mdtB</i>	Perfect	RND efflux pump	Aminocoumarin antibiotic	100	100
	<i>emrY</i>		MFS efflux pump	Tetracycline antibiotic	100	100
	<i>evgA</i>		MFS + RND efflux pump	Macrolide, fluoroquinolone, penicillin, tetracycline	100	100
	<i>mdtE</i>		RND efflux pump	Macrolide, fluoroquinolone, penicillin	100	100
	<i>H-NS</i>		MFS + RND efflux pump	Macrolide, fluoroquinolone, cephalosporin, penicillin, tetracycline	100	100
	<i>acrB</i>		RND efflux pump	Fluoroquinolone, cephalosporin, penicillin, tetracycline, others	100	100
	<i>marA</i>		RND efflux pump + porin	Fluoroquinolone, cephalosporin, monobactam, etc.	100	100
	<i>mdtG</i>		MFS efflux pump	Phosphonic acid antibiotic	100	100
	<i>acrD</i>		RND efflux pump	Aminoglycoside antibiotic	99.71	100
Antibiotic Inactivation	<i>mphA</i>	Perfect	MPH	Macrolide antibiotic	100	100
	<i>Mrx</i>		MPH	Macrolide antibiotic	100	100
	<i>TEM-1</i>		Beta-lactamase	Monobactam, cephalosporin, penicillin	100	100
	<i>EC-14</i>	Strict	Beta-lactamase	Cephalosporin	98.67	100
	<i>APH(3')-Ia</i>		APH(3')	Aminoglycoside antibiotic	98.15	100
	<i>APH(6)-Id</i>		APH(6)	Aminoglycoside antibiotic	99.64	100

Mechanism	Gene	RGI Criteria	AMR Gene Family	Drug Class	% Identity	% Length
	<i>tet(A)</i>		MFS efflux pump	Tetracycline antibiotic	99.49	94.1
	<i>Escherichia coli emrE</i>		SMR efflux pump	Macrolide antibiotic	99.09	100
	<i>emrA</i>		MFS efflux pump	Fluoroquinolone antibiotic	99.74	100
	<i>mdtH</i>		MFS efflux pump	Fluoroquinolone antibiotic	99.75	100
Target Alteration	<i>PmrF</i>	Strict	Phosphoethanolamine transferase	Peptide antibiotic	99.69	100
	<i>ArnT</i>		Phosphoethanolamine transferase	Peptide antibiotic	63.88	99.82
	<i>eptA</i>		Phosphoethanolamine transferase	Peptide antibiotic	99.63	100
	<i>bacA</i>		Undecaprenyl pyrophosphate protein	Peptide antibiotic	99.63	100
	<i>vanG</i>		Van ligase	Glycopeptide antibiotic	39.72	104.3
	<i>Haemophilus influenzae PBP3</i>		PBP mutation	Penicillin, cephalosporin	53.11	96.39
	<i>soxR</i>		ABC, MFS, RND efflux + reduced permeability	Multiple drug classes	100	100
	<i>soxS</i>		ABC, MFS, RND efflux + porin mutation	Multiple drug classes	100	100
	<i>AcrAB-ToIC + AcrR</i>		RND efflux pump	Multiple drug classes	100	100
Target Replacement	<i>sul2</i>	Perfect	Sulfonamide-resistant sul gene	Sulfonamide antibiotic	100	100
Target Protection	<i>QnrS1</i>	Perfect	Quinolone resistance protein (qnr)	Fluoroquinolone antibiotic	100	100

Shigella sonnei BCSIR-MHS-F2's considerable virulence potential is often highlighted by the bacteria's capacity to adapt to host settings, get nutrition, and elude host immune responses. Further analysis with the IslandPath-DIMOB tool revealed a diverse array of proteins, enzymes, transporters, and regulatory elements in *Shigella* BCSIR-MHS-F2 isolates; most of these were directly or indirectly related to resistance mechanisms, mobile genetic elements, and other vital bacterial processes. Using bioinformatic tools such as SIGI-HMM, which uses Hidden Markov Models to detect genomic islands, phage, and resistance genes, this work has been able to highlight several important genomic features, as shown in Fig. 4b.

A number of crucial Virulence Factor (VF) genes were found (Table 4). These include the type-I fimbrial adhesion-related proteins *fimD*, *fimA*, *fimI*, and *fimH*, which promote host cell attachment ^{21,22}. Genes involved in the synthesis and absorption of siderophores, such as *entC*, *entE*, *entF*, *fepA*, and *fepE*, represented iron acquisition systems ²³. *Shigella* BCSIR-MHS-F2 contains Type VI secretion system genes (*vgrG1*, *etpD*, *espX1*) and Type II secretion system components (*gspD*, *gspE*, and *gspF*) (Fig. 4c), which supports the bacterial ability to release pathogenic proteins into host cells ^{24,25}. Membrane and stress response elements (*ompA*, *msbA*) were involved to preserve envelope integrity and resilience to environmental stresses ^{26,27}. This bacterium also contains *hlyE* gene which encodes a hemolysin-like toxin ²⁸, and *cfaC/cfaD* which involved for colonization factor production ²⁹.

Table 04

Mapping Virulence Factors of *Shigella sonnei* BCSIR-MHS-F2. Profiling identifies virulence factors utilizing represented taxa, genes, and representation classes.

Virulence Factor Class	Virulence Factor	Virulence Gene	Represented Genera of Virulence Factor
Adherence	EaeH	<i>eaeH</i>	<i>Escherichia</i>
	CFA/I fimbriae	<i>ibeB</i>	
Autotransporter	AatA, AIDA-I type	<i>aatA</i>	
	UpaG adhesin, trimeric AT	<i>upaG</i>	
Non-LEE encoded TTSS effectors	EspL1	<i>espL1</i>	
	EspL4	<i>espL4</i>	
	EspR1	<i>espR1</i>	
Toxins	Hemolysin/cytolysin A	<i>clyA</i>	<i>Salmonella, Shigella, Escherichia</i>
Type VI secretion system	ACE T6SS	<i>ACE T6SS, aec15, aec17, aec19, aec22, aec23, aec24, aec27, aec29, aec31, aec32</i>	<i>Escherichia</i>
		<i>aec16, aec18, aec26, aec30</i>	<i>Salmonella, Escherichia</i>
Unknown	colicin B (TX380)	<i>cba</i>	<i>Escherichia</i>
	ETT2 (SS017)	<i>ECs3712, epaP, epaQ, epaS, eprJ, eprK, etrA, iagB, orgB</i>	
	etp (SS207)	<i>etpE, etpF, etpG, etpH, etpL</i>	
	Hemorrhagic Coli pili (HCP) (AI102)	<i>hofB/hcpB, hofC/hcpC, ppdD</i>	
	Stg fimbriae (AI047)	<i>stgA, stgB, stgC, stgD</i>	
	peritrichous flagella (AI140)	<i>tar/cheM</i>	
	E. coli YcbQ laminin-binding fimbriae (ELF)	<i>ycbR, ycbS, ycbT</i>	
	EHS (SS185)	<i>Z0263</i>	
	F9 fimbriae (AI090)	<i>Z2200, Z2201, Z2206</i>	
Endotoxin	LOS	<i>lpcA</i>	<i>Haemophilus</i>
Secretion system	T2SS (Type II secretion system)	<i>gspM</i>	<i>Shigella, Escherichia</i>
Immune evasion	Capsule	<i>Capsule</i>	<i>Streptococcus</i>
Others	O-antigen	<i>O-antigen</i>	<i>Yersinia</i>

Bacteriophage Diversity, Mobile Genetic Elements and Horizontal Gene Transfer

Shigella BCSIR-MHS-F2 strain consists of phage-related sequences, mobile genetic elements, recombination and repair modules, and core replication genes. According to Fig. 5a, fundamental replication and DNA maintenance genes such as, *dnaA*, *dnaE*, *ligA*, *recA*, *polA*, and *uvrABC* are present^{30,31}. Horizontal gene transfer events are controlled by transposases (*tnpA*), integrases (*int*, *xerD*), and recombinases³². Genes related to stability and defense, like *hipA*, *cpxA*, and *mukB* are present^{33,34}. Alongside, the presence of phage-related elements (*virB4*, *intD*) suggests phage integration with bacterial genome recently. Overall, *Shigella* BCSIR-MHS-F2 is a highly adaptive organism with strong potential for DNA replication, repair, stress tolerance, and genetic exchange³⁵. According to Fig. 5b, the circular genome contains 4,763 genes and it is compartmentalized with phage genes (219) and bacterial genes (4544). Six prophages contain structural and replication-related proteins, suggesting recent or possibly active integration events. These findings emphasize the important role of prophage-driven gene transfer and lysogenic conversion in *Shigella*.

The *Shigella sonnei* BCSIR-MHS-F2 contains one plasmid (~ 250 kb), which has conjugative transfer elements, replication modules, and antibiotic resistance genes (Fig. 5c). Important antimicrobial resistance genes such as *TEM-1*, *mphA*, *APH(3')-Ia*, *APH(6)-Id*, and *tetA* are present, indicating broad resistance to aminoglycosides, tetracyclines, macrolides, and β -lactam antibiotics^{36–38}. The presence of Type IV Secretion System (T4SS) cluster (*virB4*, *virB9*, *virB10*, *taxB*) and conjugative transfer genes (*tra*, *trb*) indicates the plasmid can transmit genes horizontally^{25,39,40}. Several replication initiations (*repA*) and partitioning genes (*parM*, *parA*) were found^{41,42}. Overall, the *Shigella* BCSIR-MHS-F2 plasmid is a key mobile genetic element that contributes to the dissemination of multidrug resistance within bacterial populations.

Defense Mechanisms Against Phage Infections

A strong immune architecture is also reflected in the presence of different defense systems in *Shigella* BCSIR-MHS-F2, ranging from classical restriction-modification (RM) to Cyclic-oligonucleotide-based anti-phage signaling systems (CBASS). The defensive systems consist of like nucleases (*RecB*), sensors (*GacA*), effectors (*CapV*), and related accessory proteins that together coordinate detection, signaling, and neutralization of phage attacks (Fig. 6a). Some defense systems are well-conserved and complete, while others may be partial or fragmented, suggesting evolutionary remnants or horizontal gene transfer events. The identification of multiple diverse defensive operons improves our understanding of the *Shigella* strain's potential resistance to phage infection^{43,44}.

Shigella BCSIR-MHS-F2 contains two identical CRISPR (Clustered Repeats Interspaced Palindromic Repeats) arrays, each of which has the Type IE and Type III Cas genes. Numerous Cas gene clusters (*cas1*, *cas3*, *cas5*, *cas6*, *cas7*, *cse1*, and *cse2*) are identified (Fig. 6b). The presence of CAS-Type IE Clusters and Cas Subtypes suggest a defensive potential and capable of both interference and spacer acquisition. Ancestral Type I genes (*cas13-16*) are present in the Type IE-dominant system, suggest horizontal gene transfer^{45,46}. Overall, this suggests that the strain is well adapted to its environment and evolutionarily robust, potential to better resist phage attacks and the entry of foreign genetic material^{47,48}.

Materials and Methods

Ethics Statement

The collection and use of poultry-derived samples were conducted in accordance with institutional ethical guidelines and approved by the Institutional Ethical Review Committee of Jagannath University, Dhaka (Approval Code. 52/2026 JnU ERC). No experimental procedures were performed on live poultry, and no animals were euthanized specifically for this study.

Sample Collection, Processing and Presumptive Identification

The poultry samples were collected from domestic chickens at North Jamshing Village, Jamshing, Radio Colony, Savar, Dhaka, Bangladesh. The Genomic Research Laboratory promptly received fecal samples of poultry that were collected using a sterile cotton swab and transferred to the laboratory using Cary-blair transport medium to preserve viability. Selenite F broth was employed for bacterial enrichment and incubated at 37°C for 24 hours. The following day, a sterile inoculating loop was immersed into enrichment broth, streaked into fresh XLD media, and incubated at 37°C for 24 hours. The colonies were further sub-cultured for presumptive *Shigella* isolation, as they appear red without black centers. A series of biochemical assays were conducted to facilitate presumptive identification, and the results were interpreted using the Microrao online bacterial identification tool (<https://www.microrao.com/identify.htm>).

Antimicrobial Susceptibility Test, Biofilm Formation Assessment

The antimicrobial susceptibility test was conducted using the disc diffusion procedure in accordance with the Kirby Bauer method. This strain was subjected to 14 antibiotics from various classes that were recommended by the CLSI (Clinical and Laboratory Standards Institute; CLSI) guidelines. The crystal violet (CV) biofilm formation assay was employed to evaluate the isolates' biofilm-forming capacity. In summary, 100 µL of a diluted culture of test isolate was inoculated into Thermo Scientific™ 96-Well Microtiter Microplates. 125 µL of a 0.1% crystal violet solution was used to stain the biofilm that had formed after overnight incubation at 37°C. Then, after washing crystal violet three times, 100 µL of 30% acetic acid was used for dissolving the biofilm coated bacteria in well. The UV absorbance at 570 nm was measured using a SpectraMax microplate reader to quantify biofilm formation. The following criteria were employed to classify the isolates' biofilm-forming capabilities: $OD \leq OD_{cut}$ (non-biofilm), $OD_{c} < OD \leq 2 \times OD_{cut}$ (weak biofilm), $2 \times OD_{cut} < OD \leq 4 \times OD_{cut}$ (moderate biofilm), and $OD > 4 \times OD_{cut}$ (strong biofilm). In this context, OD_{cut} is calculated as OD_{avg} (average of OD's) of the negative control plus 3× the standard deviation of ODs of the negative control, where OD denotes the optical density of the sample in the CV assay. All assays were performed in triplicate.

Whole Genome Sequencing, Annotation and Identification

The manufacturer's protocol (NEB#T3010) was followed to extract genomic DNA from the selected isolate using the Genomic DNA Purification Kit for Gram negative bacteria (New England Biolabs, UK). WGS sequencing was performed at BCSIR using the Illumina MiniSeq Platform. FastQC (v0.11) was used to quality control of the raw FASTQ files produced from the platform⁴⁹. To delete adaptor sequences and low-quality sequences, Trimmomatic (v0.39) was used⁵⁰. After trimming, the high-quality readings were assembled de

novo using SPAdes (v3.15.4)⁵¹. The assembled genome was annotated using multiple tools, including Prokka⁵², RAST Server¹⁸, and the NCBI's Prokaryotic Genome Annotation Pipeline (PGAP)^{53,54}. The Proksee server was employed to generate a graphical representation of the genome⁵⁵. The whole genome-based identification of the isolate was performed utilizing k-mer based algorithms through the LINbase server⁵⁶ and the KmerFinder tool from the Center for Genomic Epidemiology. The Average Nucleotide Identity (ANI) was calculated to further validate the identification⁵⁷. The Centre for Genomic Epidemiology's MLST 2.0 application was employed to perform MLST typing⁵⁸.

Genome Assembly

High-quality reads were assembled into a draft genome using the integrated assembly pipelines of the BV-BRC version 3.56.67⁵⁹ and the EDGE bioinformatics platform⁶⁰. These services utilize robust algorithms like SPAdes⁶¹ to perform contig generation, scaffolding, and quality assessment.

Functional Annotation and Metabolic Profile Analysis

Subsystems were analyzed by annotating the genome sequence using the RAST server¹⁸. The COG and KEGG analysis was retrieved to investigate metabolic pathways from the Type Strains Genome Database (<https://gctype.wdcm.org/>)^{62,63}. In addition, the Prokka⁵² and ShinyGO v0.81 tools were employed to conduct gene enrichment and network analysis for pathway analysis⁶⁴.

Pangenome and Pathogenicity Analysis

Pangenome and pathogenicity analyses were conducted on all thirty-two genomes (n = 32) of *Shigella sonnei*, including the isolate used in this study. The analysis via the Integrated Prokaryotes Genome and Pan-genome Analysis tool IPGA v1.09⁶⁵ confirmed that there is a high degree of genetic relatedness between *Shigella sonnei* BCSIR-MHS-F2 and other *Shigella sonnei* strains. The genome sequences were selected based on the availability of the following information: isolation country, MLST 656, geographic group, collection year, and high genome quality. Following the screening process, 32 genomes from seven distinct countries were downloaded in FASTA format from the BV-BRC server⁶⁶ and annotated using Prokka⁵². Subsequently, the gff3 files were subjected to pangenome analysis using Roary⁶⁷, a tool that swiftly constructs large-scale pan genomes and identifies the core and accessory genes. PathogenFinder (v1.1⁶⁸) was employed to conduct pathogenicity profiling. The Tidyverse package in RStudio was used to analyze and visualize the resulting data⁶⁹. PathogenFinder evaluates input data from proteomes, genomes, or raw sequencing reads to predict bacterial pathogenicity, providing insight into the probability of an organism being pathogenic to humans.

Antimicrobial Resistance Genes, virulence factor Genes and Virulence Island Investigation

The CARD server⁷⁰ was employed to investigate antibiotic resistance genes (ARGs). Only the flawless and strict hits were considered, and low-quality reads or loose hits were excluded from the analysis. Virulence Factors of Bacterial Pathogens Database (VFDB)⁷¹ was employed to identify the virulence factor genes (VFGs). This web-based platform is a comprehensive, manually curated resource that serves as an integrative resource for the analysis of virulence factors in pathogens that cause infectious diseases in humans and animals. The

metal resistance genes were investigated using BacMet ⁷², a bioinformatics platform that is user-friendly and specifically designed for the identification and analysis of antibacterial biocide and metal resistance genes. The core database for the analysis was the manually curated database of genes with experimentally confirmed resistance function of BacMet. Virulence factors and pathogenic islands were visualized using the dedicated analysis pipelines available within IslandViewer and EDGE Bioinformatics.

Identification of Mobile Genetic Elements and Bacteriophages

PlasmidFinder tool ⁷³ and MOB-suite ⁷⁴ were employed to conduct the initial plasmid analysis. The Enterobacteriales database was selected for the PlasmidFinder utility, and the minimum percentage identity and minimum percentage coverage were set to 95% and 60%, respectively. Additionally, the MOB-Recon algorithm was implemented within the MOB-suite to investigate the plasmids. The input was the bacterial genome sequence in FASTA format. The analysis was conducted with a minimum length of 1000 contigs. The minimum sequence identity and minimum E-value were established at 80% and 1e-05, respectively.

The VirSorter tool ⁷⁵ and the PHASTEST server ⁷⁶ were employed to conduct the prophage investigation. The IntegronFinder tool was employed to investigate the isolate's integron presence ⁷⁷). The MobileElementFinder tool ⁷⁸ and the mobileOG-db database ⁷⁹ were employed to analyze the mobile genetic elements (MGEs). The PHASTEST (PHAge Search instrument with Enhanced Sequence Translation) was employed to investigate the integration of phages into the bacterial genome ⁸⁰.

Prediction of Antiviral Defense Systems

Bacterial defense systems against viral infections, such as CRISPRCas and abortive infection systems, were identified using a suite of bioinformatics tools: DefenseFinder44, PADLOC v2.0.045, and CRISPRCasFinder 4.2.2046.

Discussion

This study investigates the antimicrobial susceptibility, biofilm formation and whole genome analysis of *Shigella sonnei* BCSIR-MHS-F2 isolated from poultry in Bangladesh. In WGS analysis, this study focused on AMR genes, virulence factors, mobile genetic elements, plasmid content, and pan-genomic organization of *Shigella*.

Shigella sonnei BCSIR-MHS-F2 is showing sensitive against Ceftazidime, Ciprofloxacin, Amikacin, moderate against Tazobactam, Gentamicin, and resistant against Amphotericin, Ceftriaxone. This isolate is shown to be moderate biofilm former bacteria. Those findings are similar to the outcome of Clinical *Shigella* infections. For whole genome sequencing, high-quality DNA is mandatory. For initial bacterial identification, 16S rRNA amplification was performed PCR ⁸¹. The 16S rRNA gene is a conserved genetic marker that is widely utilized for phylogenetic investigations and bacterial identification ⁸². Gel electrophoresis showed clear amplicon bands, confirming that the universal 16S primers worked well and the DNA from *Shigella sonnei* BCSIR-MHS-F2 was of good quality. This ensured that the DNA was properly extracted and reliable for future sequencing.

Shigella BCSIR-MHS-F2 has a genome size of 5.04 Mbp and a GC content of 50.58%, which aligns with typical *Shigella* characteristics and supports its classification within the genus ^{83,84}. A total of 5,106 coding sequences (CDS) and 502 hypothetical proteins were found, which indicates genetic complexity ^{83,84}. In genome

annotations, the findings revealed that significant genes are involved in metabolism, cellular functions, and pathogenesis. Notably, many genes were linked to stress response (198), DNA replication and repair (156), and membrane transport (109), highlighting the bacterium's ability to adapt to host and environmental stresses. Horizontal gene transfer events are demonstrated by mobile genetic elements, including transposases and insertion sequences ⁸⁵.

In the phylogenetic tree, BCSIR-MHS-F2 clusters closely with both *S. sonnei* and *S. boydii*. The species identification was further confirmed by dDDH values above 80% and ANI values over 96%. The genomic clustering further supports the idea that *Shigella* strains originated from different *E. coli* lineages that independently gained virulent traits ⁸⁶.

Genomic analysis of *Shigella sonnei* is important in places like Bangladesh, where humans, animals, and the environment closely interact. Pangenome analysis shows that *S. sonnei* has an open genome, with a core genome of around 2,500 genes and a total pan-genome exceeding 11,000 genes. An open pangenome allows *Shigella* to be genetically flexible and acquire strain- or niche-specific genes. The bacterium is constantly evolving by gaining new genes from phages, transposons, and plasmids. *Shigella* BCSIR-MHS-F2 consists of gene clusters to the accessory genome which involved iron absorption ^{87,88}, antibiotic resistance ^{89,90}, and environmental stress tolerance ⁸⁷. Rarefaction curves further confirmed that the pan-genome is not yet fully saturated ^{91,92}. This highlights the continuous genomic surveillance of *Shigella* ⁹³. Gene cluster analysis showed that BCSIR-MHS-F2 shares gene families with *S. sonnei*, *S. flexneri*, and *E. coli* K-12. These clusters involved with membrane transport systems, DNA repair, and fundamental metabolic processes ^{84,94}. Importantly, resistance and virulence gene clusters were often located in unique, strain-specific regions, highlighting that they were likely acquired through horizontal gene transfer ⁹⁵. In contrast, strains such as *E. marmotae* and *E. ruysiae* had more unique gene clusters, suggesting they adapted to specific niches and followed more distant evolutionary paths ⁹⁴. The study showed that *Shigella* is closely related to *E. coli* both phylogenetically and functionally, blurring taxonomic lines and emphasizing the role of genomic context in classifying and monitoring pathogens ^{96–98}.

Shigella BCSIR-MHS-F2 genome contains many antibiotic resistance genes (ARGs) across multiple drug classes. Notably, the *blaTEM-1* gene, which produces beta-lactamase and provides resistance to beta-lactam antibiotics, is present ^{99,100}. Tetracycline resistance is mediated by *tetA*, *tetR* and macrolide resistance by *mphA* ^{99,101}. The genome also contains aminoglycoside-modifying enzymes (*aadA*, *APH(3')-Ia*) and sulfonamide resistance genes (*sul2*) ¹⁰², suggesting multi-class resistance ^{101,102}. The genome also reveals efflux pump systems (*acrB*, *emrY*, and *mdtB*), which defines the non-specific resistance mechanisms that enable the bacterium to extrude antibiotics ¹⁰³. These findings agree with previous studies on multidrug-resistant *Shigella*, which often carry both chromosomal efflux systems and plasmid-based resistance genes ^{104,105}.

The virulence profile of *Shigella* BCSIR-MHS-F2 consists of key genes for adhesion (*fimA*, *fimH*), iron uptake (*entC*, *fepA*, *fhuA*), serum resistance (*ompA*), toxin production (*clyA*), and type II and type VI secretion systems. These features indicate strong colonization and immune evasion, highlighting the *Shigella*'s possible risk of spreading from animals to humans ^{106,107}. The plasmid carries conjugation genes (*traG*, *tral*, *virB*) and antibiotic resistance genes (*blaTEM-1*, *tetA*, *sul2*, *mphA*), indicating it can easily transfer genes to other bacteria. When resistance and virulence genes are located on the same plasmid, antibiotic pressure can select for both

together, speeding up the spread of multidrug-resistant infections^{108–110}. PHASTEST identified six complete prophage regions containing genes like integrases, lysins, and tail fiber proteins. These prophages can help move genes between bacteria and increase genome diversity. The close location of some virulence genes to prophage insertion sites suggests possible phage-mediated gene transfer^{111,112}. In *Shigella*, prophages have played an important role in gaining toxin genes and other virulence factors, making them key contributors to bacterial evolution¹¹³. Using the PADLOC tool, several antiviral defense systems were identified in the *Shigella* BCSIR-MHS-F2 genome, including restriction-modification (RM) systems, CBASS, and abortive infection systems like Shedu and Gabija. These systems help bacteria survive by blocking invading phage DNA and strengthening defense at the population level. Two CRISPR-Cas systems (type I-E and type III-A) with unique spacer sequences were also identified. These systems help the bacteria remember past phage infections and respond more effectively. The balance between gaining new genes (through horizontal gene transfer) and maintaining stability (via CRISPR-Cas) reflects *Shigella*'s complex evolutionary strategy⁹⁰.

One major public health concern is the detection of a drug-resistant *Shigella* strain in poultry, which poses a zoonotic risk, especially in settings with poor biosecurity and close human–animal contact. Poultry can act as a reservoir for antimicrobial resistance (AMR), particularly when antibiotics are used improperly, allowing resistance to spread and increase¹¹⁴. Treatment becomes more difficult, especially in low-resource settings, because resistance and virulence genes can spread through plasmids and prophages. This increases the chance of these genes being transferred to human-associated bacteria^{115,116}. CRISPR-Cas systems also show that this strain is highly adaptable and can persist over time. To reduce these risks, steps such as enforcing proper antibiotic use, promoting probiotic alternatives, strengthening AMR surveillance, educating poultry farmers, and expanding whole genome sequencing (WGS) diagnostics are recommended. Effective AMR control needs a One Health approach that links animal, human, and environmental health efforts. Overall, this study shows that *Shigella* BCSIR-MHS-F2 has strong resistance and virulent abilities. These findings stress the need for public awareness, proper regulations, and coordinated surveillance to reduce AMR risks and protect both human and animal health.

Conclusion

This study provides genomic evidence of the potential role of poultry as a reservoir of antimicrobial-resistant and zoonotic *Shigella*. Whole-genome sequencing of *Shigella sonnei* BCSIR-MHS-F2, isolated from Crocker chicken, revealed a highly adaptable genome carrying antimicrobial resistance and virulence-associated genes, plasmids, prophages, and other mobile genetic elements. Its close genomic similarity to human-associated *S. sonnei* strains suggests a potential risk of zoonotic spillover and cross-species transmission. The open pan-genome structure further highlights the adaptive capacity of *Shigella* to acquire novel traits. These findings underscore important public health concerns associated with poor biosecurity and intensive antibiotic use in poultry production. Strengthened biosecurity, responsible antimicrobial use, genomic surveillance, and farmer education within a coordinated One Health framework are essential to mitigate these risks and protect animal and human health.

Declarations

Competing interests

The authors declare no competing interests.

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Author Contribution

FIMM contributed to the study design and experimental work. NU and MMHS performed genomic and bioinformatics analyses. NU, FIMM, and SMA analyzed the data and drafted the manuscript. SFC, SRN, and BG conducted sample preparation and genome extraction. SA, TAB, and MI managed sample preparation and sequencing. MMHS and MRI conceived the study, supervised the project, managed the data, and finalized the manuscript. All authors reviewed and approved the final manuscript.

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Data Availability

The genome data of *Shigella* BCSIR-MHS-F2 analyzed in this study has been deposited in the National Center for Biotechnology Information (NCBI) GenBank database under accession number JBZWKF000000000 (<https://www.ncbi.nlm.nih.gov/nuccore/JBZWKF000000000>). The raw sequencing reads have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1468195, BioSample accession number SAMN60274355, and SRA accession number SRR38949206. All datasets are publicly available through the NCBI repository.

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Figures

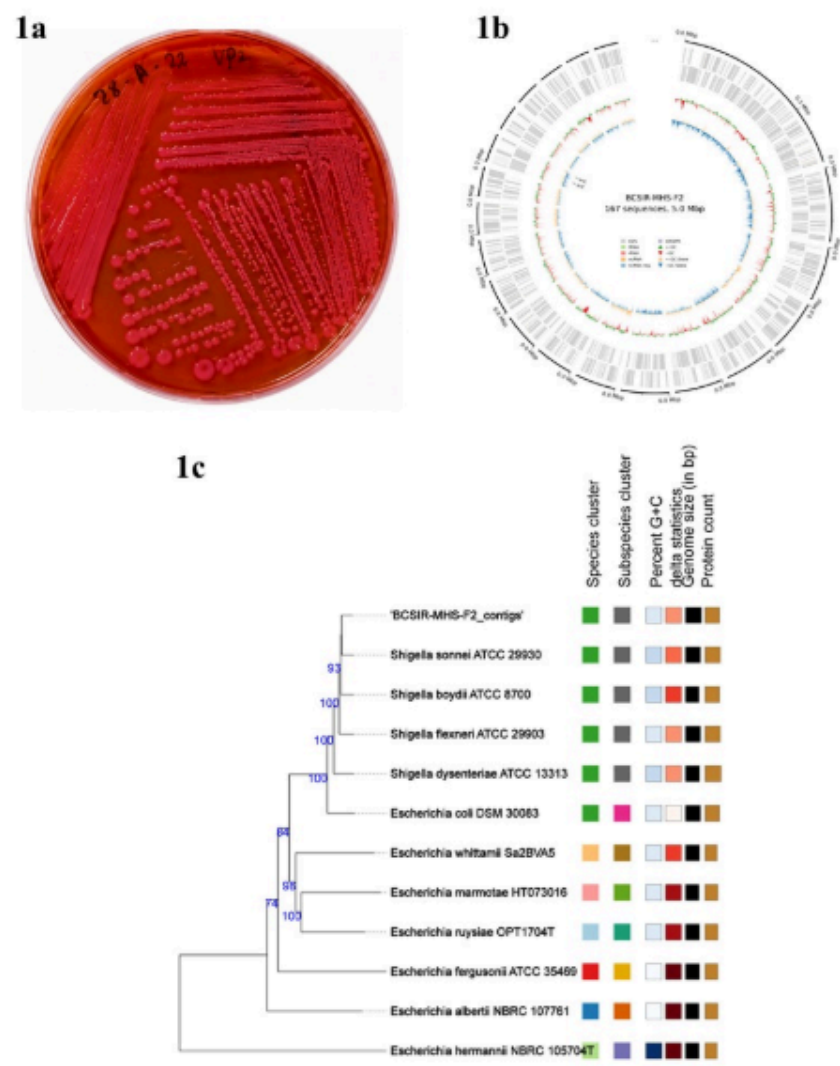


Figure 1

Bacterial growth, Genome Assembly and phylogenetic placement of *Shigella sonnei* BCSIR-MHS-F2. Initial genomic characterization confirms the species identity of the isolate. (a) In subculturing techniques, a sterile inoculating loop was used to transfer the mixed colony into freshly prepared XLD plate, and finally a single colony was found which is pink without black center, assumed to be *Shigella* strain. (b) Circular genome map of the draft assembly (5.00Mbp, 167 contigs) annotated with Bakta, showing from outer to inner: COG functional

classifications of CDS on forward and reverse strands, RNA genes, GC content (black), and GC skew (purple/green). **(c)** TYGS based phylogenetic tree indicating the evolutionary position of BCSIR-MHS-F2, highlighted in red, within the genus *Shigella*. It clustered together with the two reference strains, namely, *Shigella sonnei* ATCC 29930 and *Shigella boydii* ATCC 8700.

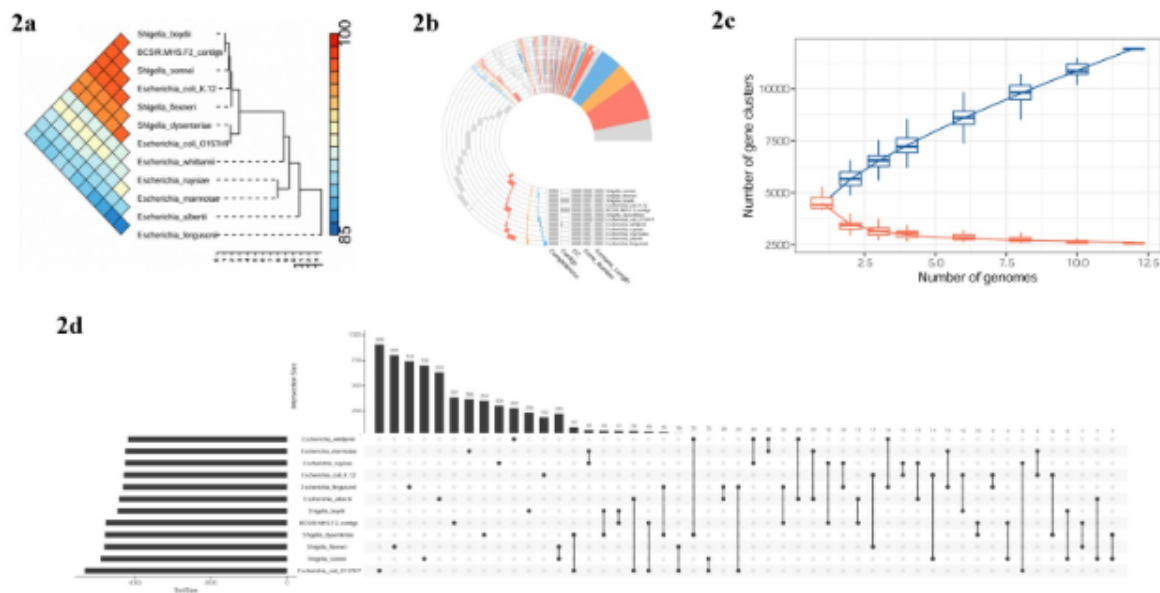


Figure 2

Phylogenetic Analysis, Core and Pan-Genome Rarefaction and Genome Cluster Intersection of a distinct *Shigella sonnei* lineage. **(a)** Heatmap depicting Average Nucleotide Identity values, which indicated that BCSIR-MHS-F2 have a very high ANI with *Shigella boydii* and *Shigella sonnei*. This places BCSIR-MHS-F2 in the *Shigella* species group, indicating a high genetic similarity and probably a common evolutionary origin. **(b)** The pan-genome is the whole set of genes found in many strains. Alongside, the core (conserved) and accessory genes (variable) also depict here. **(c)** An intersection analysis of gene clusters from several strains of *Escherichia* and *Shigella*. The blue line indicates the pan-gene, and the red line indicates the core genes. As the number of genomes rises, the core gene declines and pan-gene increases. **(d)** Gene Cluster Intersection Analysis of *Shigella* BCSIR-MHS-F2 and Related *Shigella* Strains that highlights genomic diversity, species-level similarities, and evolutionary links while summarizing both unique and shared gene families.

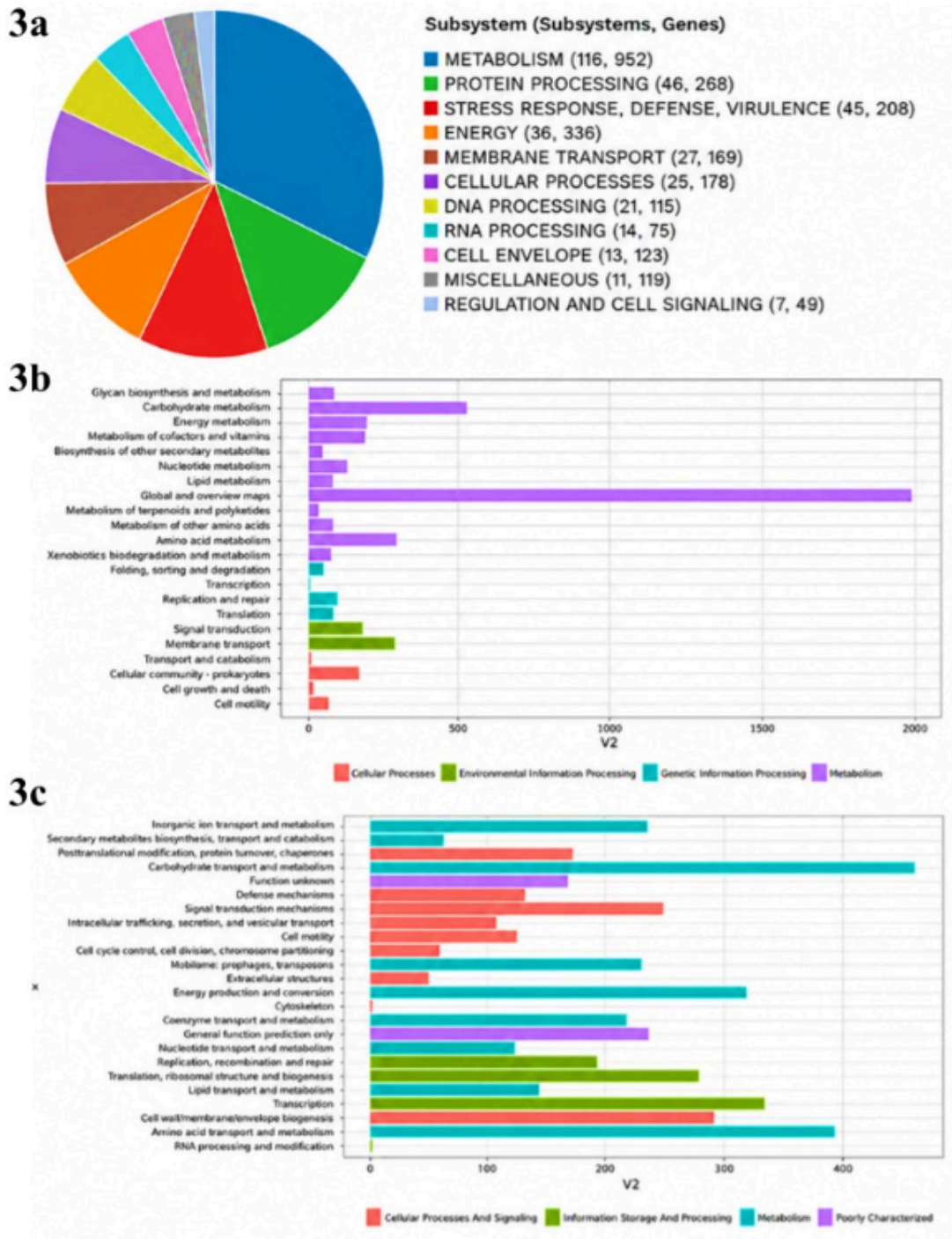


Figure 3

Functional annotation and metabolic profiling of the *Shigella sonnei* BCSIR-MHS-F2 genome. The genome is enriched with metabolic functions and pathways related to environmental adaptation. **(a)** Bar plot representing the distribution of protein-coding sequences across 11 major functional subsystems as annotated by RASTtk. **(b)** Chart of the Clusters of Orthologous Groups (COG) functional classification, showing strong representation in Metabolism, and Environmental Information Processing. **(c)** Histogram of KEGG pathway mapping, which shows the counts of genes in major pathway categories and enrichment in metabolic pathways.

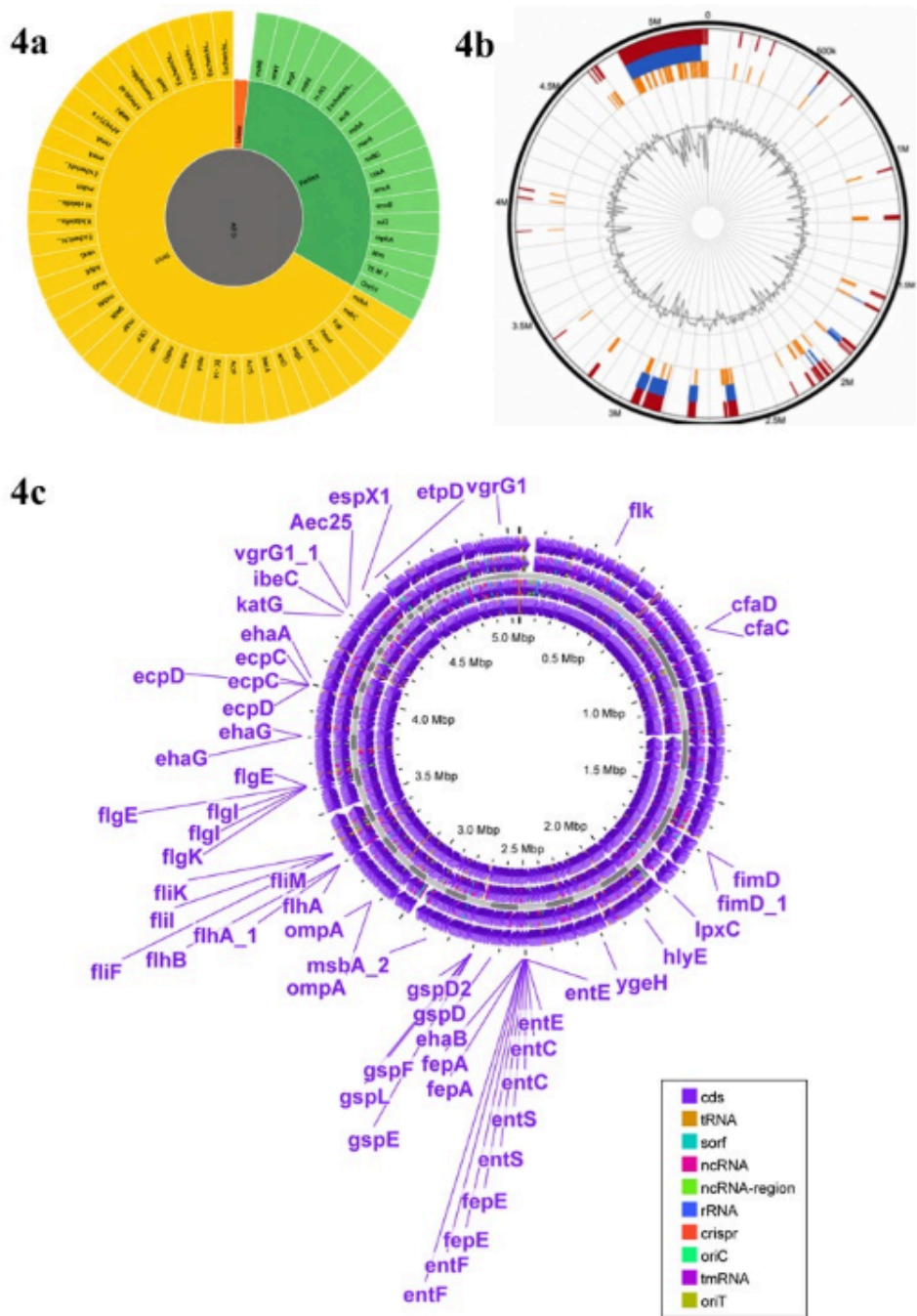


Figure 4

Antimicrobial Resistance Profiling, Genomic Islands and Virulence factors of *Shigella*BCSIR-MHS-F2. The genome harbors a diverse arsenal of virulence factors, virulence genes, and horizontally acquired elements. **(a)** Antibiotic Resistance Ontology (ARO) Distribution Reveals Dominant Resistance Genes & Hosts. **(b)** Circular genome map showing genomic islands as predicted by IslandPath-DIMOB and SIGI HMM methods indicating putative horizontal gene transfer events. **(c)** The virulence factors (VF) genes mapping. These genes are important markers of the strain's pathogenesis.

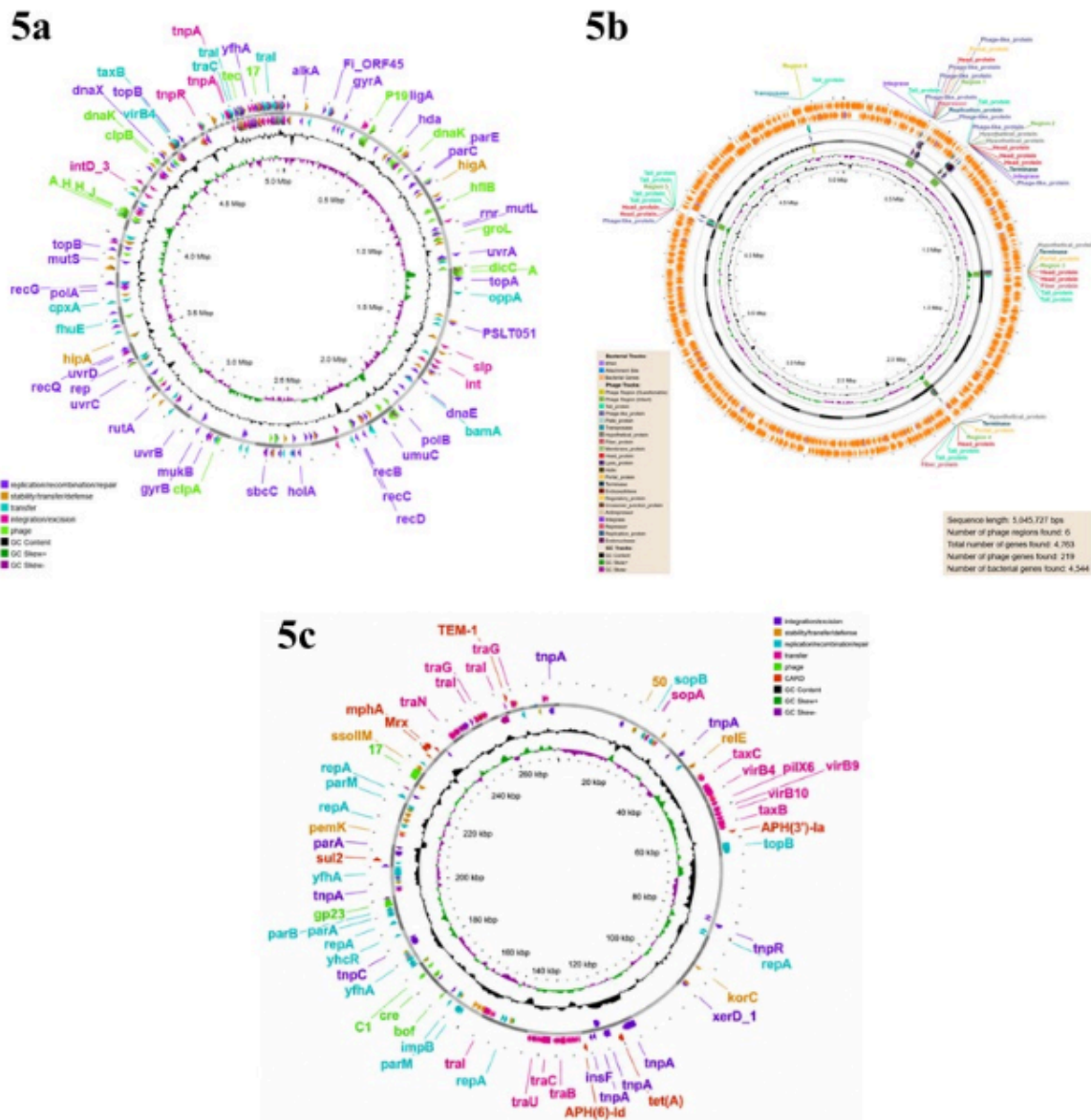


Figure 5

Mobile genetic elements and bacteriophage diversity in *Shigella sonnei* BCSIR-MHS-F2. The genome is equipped with a rich repertoire of mobile elements, including prophages and plasmids. **(a)** *Shigella sonnei* BCSIR-MHS-F2's mapping shows genes related to defense, stability, replication, repair, integration, and transfer. **(b)** Circular representation of the genome showing the location, size and completeness of six identified prophage regions. **(c)** Schematic of detected plasmid replicons, highlighting their associated antibiotic resistance genes and virulence factors.

