Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2025.118

RESEARCH ARTICLE

Genetic outlook of Colistin resistant *Salmonella enterica* Serovar Typhimurium recovered from Poultry-Environment Interface: A One Health Standpoint

Sara Mahmood, Muhammad Hidayat Rasool, Mohsin Khurshid and Bilal Aslam*

Institute of Microbiology, Government College University Faisalabad, Faisalabad 38000, Pakistan
*Corresponding author: drbilalaslam@gcuf.edu.pk

ARTICLE HISTORY (24-795)

Received: December 5, 2024
Revised: January 26, 2025
Accepted: February 5, 2025
Published online: February 27, 2025

Key words:
Comparative Genomics
Environment
Pathogen
Poultry
Salmonella enterica
Virulence Genes
WGS

A B S T R A C T

Salmonellosis is a significant public health concern globally, primarily caused by the consumption of environmentally driven contaminated food and water. *Salmonella enterica* can infect human and animals via associated environment, that makes this bacterium one of the potential one health pathogens. Regrettably, irrational antimicrobial use (AMU) in food producing animals (FPAs) like poultry creates selection pressure that leads to the emergence of resistance against critically important antibiotics for human medicine such as colistin. In this study, *Salmonella enterica* serovar Typhimurium isolates were recovered from poultry and their associated environments. Confirmation and antibiotic resistance profiling of the isolates was done through standard microbiological procedures and clinical and laboratory standards institute (CLSI) guidelines. Whole-genome sequencing (WGS) was employed to assess the genomic features of the *S. enterica* isolates, with a focus on identifying antimicrobial resistance and virulence genes. Phylogenetic relationships were analyzed through comparative genomics and whole-genome multi-locus sequence typing (wgMLST). The results revealed a significant distribution (23%) of *S.* Typhimurium, whereas 8% among them were colistin resistant. The isolated strain exhibited unique genetic outlook compared to previously reported genomic characteristics. Notably, unlike other *S. enterica* genomes from poultry-related environments, this isolate contained a complete phage (Sal3) integrated into its genome. Several virulence genes and antibiotic resistance genes (ARGs) were identified, responsible for the distinct pathogenic ability of *S. enterica*. The present study displayed a detailed genomic vista of *S. enterica*, that may be useful for the future interventions to control the infections caused by this one health pathogen.

To Cite This Article: Mahmood S, Rasool MH, Khurshid M, Aslam B, 2025. Genetic outlook of Colistin resistant *Salmonella enterica* Serovar Typhimurium recovered from Poultry-Environment Interface: A One Health Standpoint. Pak Vet J, 45(1): 246-256. http://dx.doi.org/10.29261/pakvetj/2025.118

INTRODUCTION

Salmonella enterica is a leading cause of bacterial foodborne illnesses worldwide. It is a recognized as one health pathogen that contributing significantly to both mortality and morbidity, especially in children under five years old, as well as causing substantial economic losses (Jain *et al.*, 2020). With over 2,600 serotypes identified, *S. enterica* is classified based on a combination of 146 lipopolysaccharide antigens and 114 flagellar proteins. Among the non-typhoidal *Salmonella* (NTS) serovars, those most pathogenic to humans include *S.* Typhimurium, *S.* Enteritidis, *S.* Newport, and *S.* Heidelberg (Ali and Alsayeqh, 2022). These serovars are typically transmitted through contaminated food, water, and direct contact with infected meat and poultry. Globally, the World Health Organization (WHO) estimates that there were approximately 150 million foodborne infections caused by NTS. The Centers for Disease Control and Prevention (CDC) reports around 9 million annual foodborne infections, of which more than 1 million are attributable to NTS (Gutierrez *et al.*, 2020). Furthermore, *S. enterica* has been identified as a major cause of foodborne illnesses in China and across many European countries, affecting both humans and poultry (Lamichhane *et al.*, 2024). These infections persist in both developed and developing nations, posing a serious threat to socioeconomic development and contributing to high mortality rates in children in low-income regions (Altaf Hussain *et al.*, 2020).

The pathogenicity of *Salmonella* is attributed to various virulence factors located on both chromosomes and plasmids. The key virulence genes of *S.* enterica include *invA, hilA, ipfA, sivH, sefA, sopE,* and *agfA*. These genes are often clustered in regions on the chromosome known as *Salmonella* Pathogenicity Islands (SPIs), which are critical for the bacterium's virulence cycle (Sedrakyan *et al.,* 2022). SPIs 1 through 5 are found in most *Salmonella* serovars, while other SPIs are less widely distributed. Each SPI plays a distinct role in bacterial pathogenesis. For example, SPI-1 and SPI-2 are involved in macrophage apoptosis and host cell invasion, aided by the *invA, hilA,* and *sivH* genes. Systemic infections are facilitated by the *sopE* gene, while survival in macrophages is linked to *ipfA* (Kong-Ngoen *et al.,* 2022). The *agfA* gene contributes to the secretion of toxins that induce macrophage apoptosis, and *sefA* encodes proteins essential for the type-III secretion system (Wang *et al.,* 2020).

The tropical climate of Pakistan provides favorable conditions for the growth of foodborne bacteria (Khan *et al.,* 2021). Contaminated food, particularly poultry and poultry products, is a major source of disease, primarily due to unhygienic handling practices (Yousafzai *et al.,* 2019). Poultry plays a significant role in the economy, with most farms located in rural areas, extending from Punjab to Sindh. These farms produce approximately 1.3 billion broilers, 49 million layers, and 12 million breeding stock annually, making Pakistan one of the largest poultry producers globally (Shakir *et al.,* 2021). Due to the excessive and irrational Antimicrobial usage (AMU) in the Pakistani poultry industry, *S. enterica* has developed resistance to commonly used antibiotics such as ampicillin (65%), erythromycin (100%), tetracycline (88%), colistin (95%), and ciprofloxacin (84%), which are employed both as therapeutics and growth promoters (Fatima *et al.,* 2023).

Salmonellosis in poultry farms, caused by resistant microbes, results in reduced growth rates, lower egg production, increased chick mortality, and infertility in birds. The mortality rate can escalate to 80%-100% due to infected chicks, as well as the consumption of contaminated poultry meat and egg. Transmission from farm to fork occurs through the use of poultry droppings as fertilizer for crops and the discharge of effluent into rivers (Lazăr *et al.,* 2019) .

In this study, colistin resistant *S. enterica* serovar Typhimurium was isolated from poultry-environment and characterized by WGS and several bioinformatics tools. Insights into phylogenetic patterns, evolutionary relationships, comparative genomics, resistance profiling and virulence factors will be crucial for understanding the pathogenic behavior of *S. enterica* and it would provide elementary data on the control and regulation of this pathogenic bacteria related to poultry.

MATERIALS AND METHODS

Ethical approval: Approval from the Institutional Review Board (IRB) and Ethical Review Committee (ERC) (Ref No. GCUF/ERC/137, dated 3 February 2023) at the Government College of the University Faisalabad was taken for this study. Prior permission and written consent were taken for sample collection from all the stakeholders. One Health AMR laboratory (OH-AMR lab) at the Institute of Microbiology, Government College, University Faisalabad involved in this research.

Collection of samples: The samples of *S. enterica* were collected from commercial poultry farms and poultry markets between 2022 and 2023. Among 361 samples, 40 samples were from poultry meat, 40 samples each from cloacal swabs and poultry droppings, 241 samples were from the poultry environment including farm waste/litter (n=40), eggshells (n=26), feed room (n=25), farm wastewater (n=30), fluff samples (n=30), carcass (n=30), drinking water (n=30) and bird cages swabs (n=30). Poultry meat of 25g were collected for each sample aseptically, while sterile swabs were used to collect samples from the fresh droppings, cloacal, and environmental sources. Collected samples were stored at 4ºC and transported to the laboratory in sterile containers for further processing.

Isolation of *Salmonella enterica:* The samples (25g) of each suspended in the 225mL of buffered peptone water (Merck Germany) for pre-enrichment which followed by 24-h incubation at 37°C. A serial dilution of each sample was made up to 10^{-8} after the completion of enrichment. All other types of Gram-negative bacteria eliminated by the pre-enriched inoculum (0.1mL) cultured in 10mL Rappaport-Vassiliadis (RV) broth (Merck, Germany) for consecutive incubation at 42°C for 16–18h. A loop full of enriched sample streaked on Xylose-Lysin-Deoxycholate Agar (XLD; Merck, Germany) and incubated at 37°C for 24-h. Colonies of *Salmonella* were selected and incubated on nutrient agar plates (Oxoid, Hampshire, England, United Kingdom) at 37°C for 24h.

***S.* Typhimurium confirmation:** Confirmation of the isolates was done through PCR by using specific primers (ST-4497-F-AACAA CGGCTCCGGTAATGA; ST-4497-R-TGACA AACTCTTGATTCTGA) as described previously (Kim *et al.,* 2006). Gene Jet Genomic DNA purification kit k0722 (Thermo Scientific™) was used for the extraction of DNA as followed by the manufacturer's instructions. Briefly, Total 25µL of the PCR reaction mixture was prepared that contained 4µL of sample DNA, 8µL of PCR master-mix (Green DreamTaq, Thermo Fisher Scientific®, USA), 2µL primers (F&R), and 11µL of Nuclease-free water (Thermo Fisher Scientific®, USA). After the initial denaturation of 03 minutes PCR reaction was carried out for 35 cycles comprising denaturation for 50 sec at 94°C, annealing for 40 sec at 53°C and extension for 45 sec at 72°C with a final extension for 03 minutes at 72°C. PCR amplicons were visualized through agarose gel electrophoresis, 1.5% of the agarose gel stained with ethidium bromide was prepared and visualized in the gel documentation system (BioRad®, USA).

Antibiotic susceptibility testing: Additionally, the susceptibility of *S. enterica* isolates against different antibiotics was tested by Kirby-Baur disc diffusion method on Mueller Hinton agar (MHA) in accordance with the standard procedures recommended by the

Clinical and Laboratory Standards Institute (CLSI) (Humphries *et al*., 2021).

Confirmation for Colistin resistance: To confirm mobile colistin resistance in the isolates, phenotypic rapid polymyxin test was performed as described earlier (Vázquez *et al.*, 2022)**.** In brief, a solution (NP solution) with 0.2mg/mL concentration of colistin was made in MHB. After the addition of a pH indicator i.e. phenol red (0.0125g) the pH of this solution was set at 6.7. Afterwards, 10% D-glucose (anhydrous) was mixed in colistin solution. Prior to the onset, 5µg/150µL colistin concentration was set by adding more colistin into the solution. Bacterial suspension with 3.0 MacFarland standard was prepared and dispensed into the 96 well microtitration plate. Isolate suspensions were separately mixed with colistin free and NP solution and incubated the plate for 5 hours at 37°C.

Whole Genome Sequencing (WGS) of *S. enterica* serovar *S.* Typhimurium

Next generation sequencing: The genomic DNA of *S. enterica* isolates was extracted by using the QIAamp DNA Mini Kit, according to the manufacturer's protocol (Yang, 2019). The standard method of agarose gel electrophoresis is used to find integrity by Qubit 3.0. Fluorometric quantitation device (Life Technologies, Carlsbad, CA, USA) was used for the determination of gDNA (ng, micro/L) concentration. DNA library was prepared using the NextEra XT rapid DNA library preparation kit according to the manufacturer's instructions (Illumina Inc., USA). Sequencing of the genome performed using a MiSeq system (Illumina Inc., USA).

Analysis of *S. enterica* genome: *S. enterica* comprehensive genome analysis was done by PATRIC services, comprehensive genome analysis services tool which is based on the annotation statistics and the comparison with the other genomes of the same species. The low-quality reads were removed by trim-galore (trim galore v0.6.5.), a wrapper script to automate quality and adapter trimming as well as control the quality of the FastQ sequence by using PATRIC, a bacterial bioinformatics resource center (Davis *et al.*, 2020). The genome was assembled and contigs (FASTA sequence files) obtained by using Unicycler v0.4.8. Initial assembly was done by using de novo assemble for the short (Illumina MiSeq FASTQ) reads and then novel semi global aligner is used for the long reads. Hybrid genome assembly method is used to get high quality data and genome sequence (https://github.com/rrwick/Unicycler). Pilon v1.23 is used for the improvement and polishing of the genome by the PATRIC resource center (v3.6.12). The BV-BRC uses RAST toolkit for the annotation of the bacterial genome. It manually curated gene annotation from the SEED database (Brettin *et al.*, 2015). RAST toolkit using different algorithms and incorporated them in a pipeline of Prodigal and glimmer tools used for the search of coding sequences (CDS) (Wattam *et al.*, 2017).

Identification of plasmids, point mutations and antimicrobial resistance gene: PlasmidFinder is used for the identification of plasmids in the whole genome sequence of *S. enterica* isolate. This web tool used BLASTn algorithm and find the best plasmid with at least 80% threshold value with already available databases (Carattoli *et al.*, 2014). The visualization of the plasmids in the form of circular maps obtained by using Proksee (https://proksee.ca/), which is an expert system for assembly of genome, annotation and visualization, featuring interactive circular or linear genome maps (Grant *et al.*, 2023).

The antimicrobial resistance based on point mutations in the whole genome sequence of the *S. enterica* isolate detected with PointFinder (Zankari *et al.*, 2017). The tool BLAST the whole genome with the mutation databases and searched the best hit from it. Antimicrobial resistance genes (ARGs) in the isolated genome identified by using ResFinder (Florensa *et al.*, 2022). The web tool used BLAST to search for the resistance genes in the genomes databases.

Virulence genes detection: *S. enterica* virulence factors detected by using BLAST in the bacterial virulence factor library, PATRIC (Patho-system Resource Integration Center, www.patricbrc.org). This database contains information on virulence based on genomic functional annotations, transcriptomic experiments, protein-protein interactions and disease information already present in PATRIC database. Interaction between virulence genes on the basis of their functionality identified by STRING (https://string-db.org/). It is a database and a software for the unknown and predicted protein-protein interactions (Szklarczyk *et al.*, 2019). *Salmonella* pathogenicity islands were predicted by Islandviewer4, involve in the prediction and visualization of bacterial genomic islands using integrated, Islandpath-DIMOB and SIGI-HMM methods for its detection with precision (https://www.pathogenomics.sfu.ca/islandviewer/) (Bertelli *et al.*, 2017).

Genome enrichment analysis: Gene set enrichment (GSE) analysis extracting biological insights from whole genome sequence performed with the help of KOBAS-I (KOBAS intelligent version) tool (http://bioinfo.org/kobas/). It works in two parts "annotation module" and "enrichment module". KOBAS generates information based on multiple databases of pathways, diseases and GO information by using contigs in the genome and intelligently prioritize the best relevant biological pathway (Bu *et al.*, 2021).

Comparative and pan-genome analysis: The comparative analysis of isolated genome of *S. enterica* compared with 12 other genomes of different species of *Salmonella* collected from NCBI (Accession number of all the sequences are available in a table) to evaluate the evolutionary relationship among them. IPGA (https://nmdc.cn/ipga/) is a handy web service for pan genome analysis in prokaryotes (Liu *et al.*, 2022).

Prophage analysis: PHASTEST (Phage Search Tool enhanced release) (https://phastest.ca/) was used to find the putative prophage sequence in the isolated genome of *S. enterica*. It is used to find prophage regions as "intact"

(score>90) "questionable" (score=70-90) and "incomplete" (score<70) based on the proportion of phage genes in the isolated genome. The complete and functional prophage is classified as intact (Wishart *et al.*, 2023). The annotation of the identified phage genome was done with the help of PhageScope (Wang *et al.*, 2024).

Identification of drug targets in virulence gene and point mutation in ARG: Virulence gene *sipA* of *S. enterica* genome was selected and aligned (BLASTP) with the drug targets reported by Drug bank (https://go.drugbank.com/). A database used for drugs and *in silico* drug target identification. SwissModel (https://swissmodel.expasy.org/) software used for the prediction of *sipA* structure (Waterhouse *et al.*, 2018). PyMol tool used for the visualization of the predicted structures (https://www.pymol.org/) (Rosignoli & Paiardini, 2022). Protein structure of ARG *bla-TEM176* predicted by SwissModel and by BLAST of amino acid sequences of isolated genome sequence and wild type ARG sequence point mutations observed. The complete genome sequence of *S. enterica* has been deposited in GenBank with project number PRJNA1159180. The draft genome data associated with poultry waste has been deposited in NCBI, Sequence Read Archive (SRA) (https:// ncbi.nlm.nih.gov), with temporary submission ID: SUB14714512.

Statistical analysis: Excel (Microsoft Office 365) spreadsheets were used for different statistical analyses on the combined particulars. Analysis of Variance (ANOVA) was used to find the difference across the average of sample sources and the P<0.05 was set as significant.

RESULTS

Identification and distribution of *S. enterica* serovar Typhimurium: Overall, the distribution of confirmed *S.* Typhimurium among various sources was recorded as 23 % (84/361; Table 1), the highest distribution was observed in chicken 42.5% (P<0.05). Whereas, among these total 8% (7/84) isolates were observed as colistin resistant.

Genomic Features of *S.* Typhimurium: *S. enterica* genome was sequenced and has raw reads of 1857725 and 275.15 M bases. The assembled genome of the isolate has a length of 4898153bps, an average G+C content of 51.87% and it has 67 contigs. The taxonomy of the genome confirms that the genome belongs to super kingdom bacteria and family of *S. enterica*. This genome has 4937 protein coding sequences (CDS), 72 tRNA (transfer ribonucleic acid) and 3 rRNA (ribosomal ribonucleic acid) genes. The genome has 674 hypothetical proteins, 4263 functional proteins, 1311 proteins with enzyme commission number (EC), 1047 with gene ontology (GO) functions while 922 proteins are involved in pathway (Fig. 1A).

Plasmid identification: A total of four plasmid replicons were identified, two of them, IncQ1, IncX4, having 100% identity and IncX1 has 99% while IncI2 (Delta) has 98% identity with available database in NCBI. All these plasmids belong to *Enterobacteriaceae* family and harbored antimicrobial resistance genes (ARGs). The genomic representation of these plasmids obtained as interactive circular genome maps (Fig. 1B, 1C & 1D).

Antibiotic resistance genes & point mutation analysis: In the whole genome sequence of the *S. enterica* isolate, point mutation was found in *parC* gene where ACC converted into AGC, that modified the target of ciprofloxacin. Moreover, Details of the ARGs identified with the help of ResFinder displayed in Table 1.

Virulence genes analysis: Overall, there were 150-200 virulence genes found in the genome of *S. enterica* which are encoded by different SPIs (Fig. 2A, 2B). The virulence genes include as *invA*, *sipC*, *hilC*, *hilA*, *ssaK*, *fimA*, and *csgA* which are involved in invasion via the type III secretion system (T3SS), fimbriae formation, and biofilm development of the pathogenic bacteria. *Salmonella* Pathogenicity islands showing different virulence genes in the genome with the help of islandviewer4 (Fig. 2C).

Gene enrichment pathways identification: Biological insights were extracted from the genome with the help of gene set enrichment analysis (GSE) with KOBAS-I web tool. It explored the biological functions of genes in the whole genome of *S. enterica* and identified the pathways depending on the functionality of these genes from the KEGG database. Overall, there were 28 virulence genes involved in the infection of *S. enterica*.

Comparative and pan-genome analysis: The studied genomes were observed as closely related to the isolated genome as shown by the ANI, average nucleotide identity was 89% to 98.55% between isolated *S. enterica* genome and the other 12 strains of *S. enterica*. The cluster showed

Table 1: Distribution of *S.* Typhimurium among various samples sources of the study

Sr. No.	Specimen Category	Source of Sample	No. of Collected Samples	Distribution of *S.* Typhimurium		Colistin resistant *S.* Typhimurium	Plasmid Identified	ARGs detected in WGS	*P value
1		Chicken	40	16	42%	1	IncX4/ IncI2	mcr-1.1	<0.05
	Poultry	Cloacal swabs	40	7	17.50%	ND	IncQ1	qnrS1/ aph(3')-Ia	
		Droppings	40	8	20%	1	IncX4/ IncI2	mcr-1.1	
2		Poultry Farm waste/Litter	40	10	25%	1	IncX4/ IncI2	mcr-1.1	
		Poultry Farm wastewater	30	6	20%	2	IncX4/ IncI2	mcr-1.1	
		Feed Storeroom	25	3	12%	ND	IncX1	blaTEM-176/ dfrA14	
	Environment	Farm Carcasses	30	7	23%	1	IncX4/ IncI2	mcr-1.1	
	Samples	Cage Swabs	30	4	13%	1	IncX4/ IncI2	mcr-1.1/ tet(A)	
		Drinking water	30	2	7%	ND	IncQ1	tet(A)/ floR	
		Fluff Samples	30	8	26%	ND	IncX1	blaTEM-176	
		Egg Shells	26	4	15%	ND	IncX1	blaTEM-176	
	Grand Total		361	84	23%	7			

*Chicken Samples, Farm litter & waste (*ND= not detected).

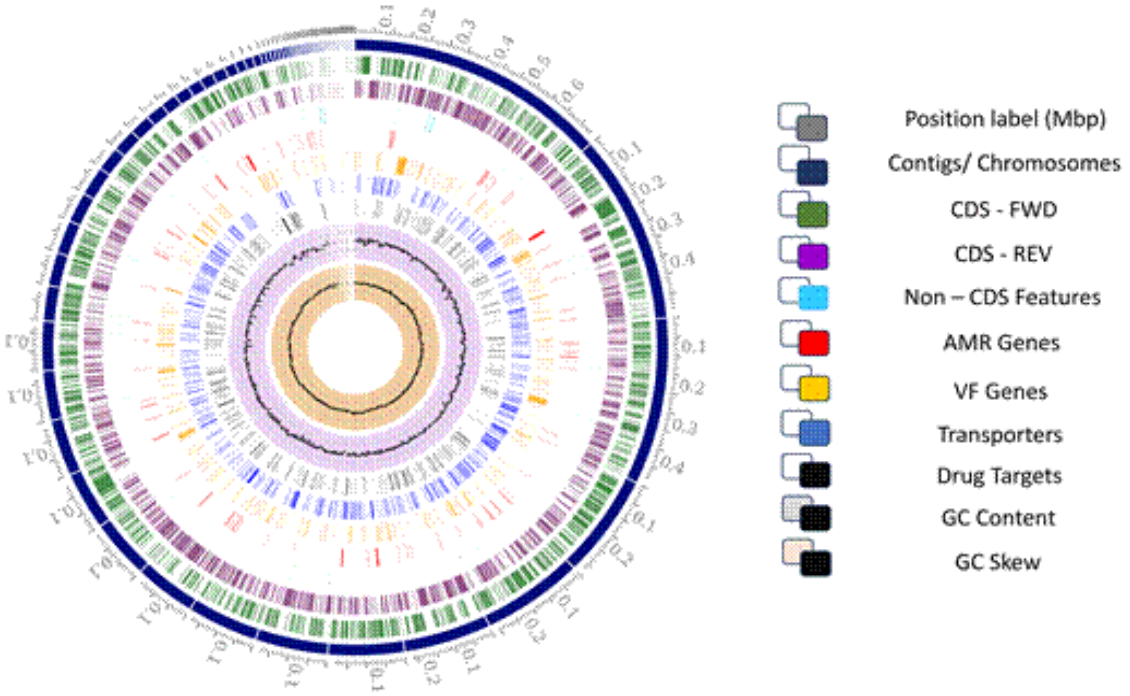

Fig. 1A: Circular Genome Map of *S. enterica*; depicting Genes Location by Functionality.

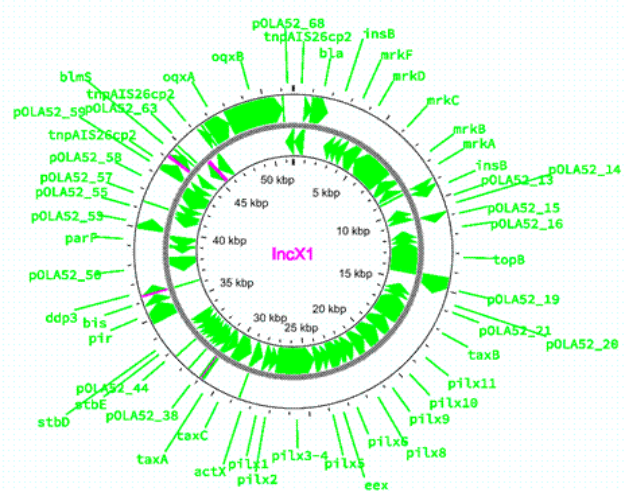

Fig. 1B: Genomic Representation of IncI2 (delta) Plasmid identified in the *S. enterica* Genome.

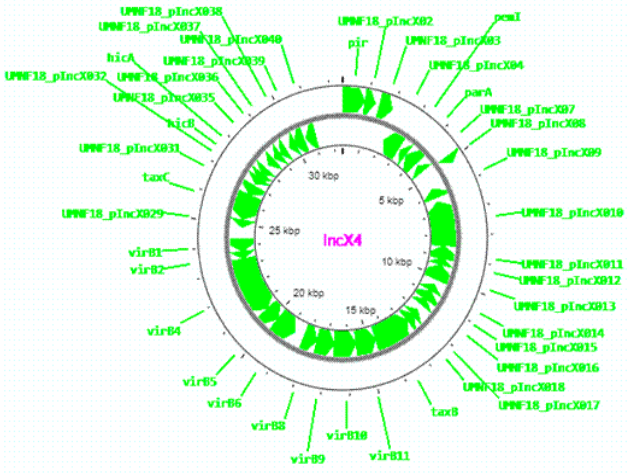

Fig. 1C: Genomic Representation of IncX1 Plasmid identified in the *S. enterica* Genome.

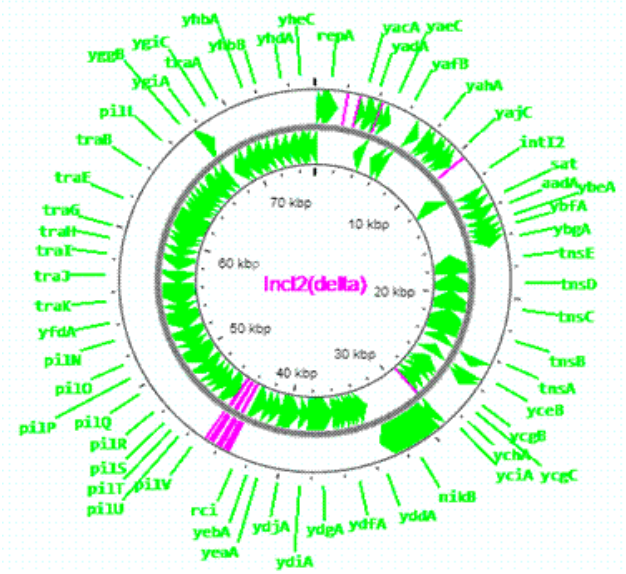

Fig. 1D: Genomic Representation of IncX4 Plasmid identified in the *S. enterica* Genome.

the unique genes in the genomes of all strains ranging from 25 to 588 while the isolated *S. enterica* genome exhibited 199 unique genes (Fig. 3A, 3B, 3C).

wgMLST analysis: PubMLST (PubMLST.org) was used for the identification of multi locus sequences in the isolated genome of *S. enterica*. The BIGSdb (Bacterial isolate genome sequence database) in which all types of data are available from bacterial single gene to complete genome. The results were further analyzed by Phyloviz in the form of a spanning tree. Each node of minimum spanning tree is sized according to the number of strains present with the corresponding sequence type (ST). Number on nodes represents sequence typing (ST).

Prophage identification: PHASTER identified two putative prophages that were classified as "incomplete" and "intact" in the studied genome. The analysis is based on the comparison of isolated *Salmonella* genome with phage genomes showing incomplete lambda phage (PHAGE_Entero_lambda_NC_001416) and intact genome sequence of sal3 phage (PHAGE_Salmon_118970_sal3_NC_031940). These bacteriophage repertoires contributed significantly to the genetic diversification of the isolated *S. enterica* genome (Fig. 4A, 4B). The functionality of annotated prophage genome showed protein involved in its integration, packaging, infection and lysis.

Mutations in predicted protein structures: The 3D Protein structures of selected genes were predicted and further analyzed for the mutations and drug targets. In particular, *bla-TEM* showed the following point mutations, V->I amino acid 82, A->V at amino acid 183 and V->A at amino acid 222. These mutations occurred in alpha-helixes and beta chain of the protein structure which involved in the maintenance of the protein conformation and its functionality (Fig. 5).

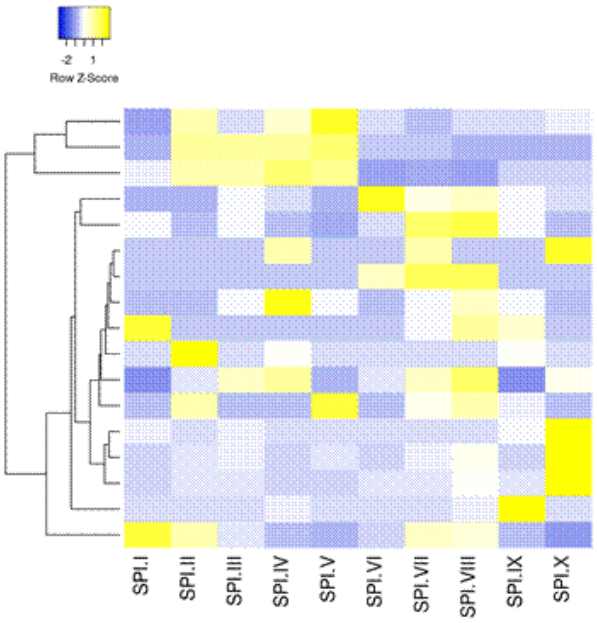

Fig. 2A: Expression of Virulence genes in *Salmonella* Pathogenicity Islands with the help of Heatmap. Pathogenicity decreases from yellow color to blue color.

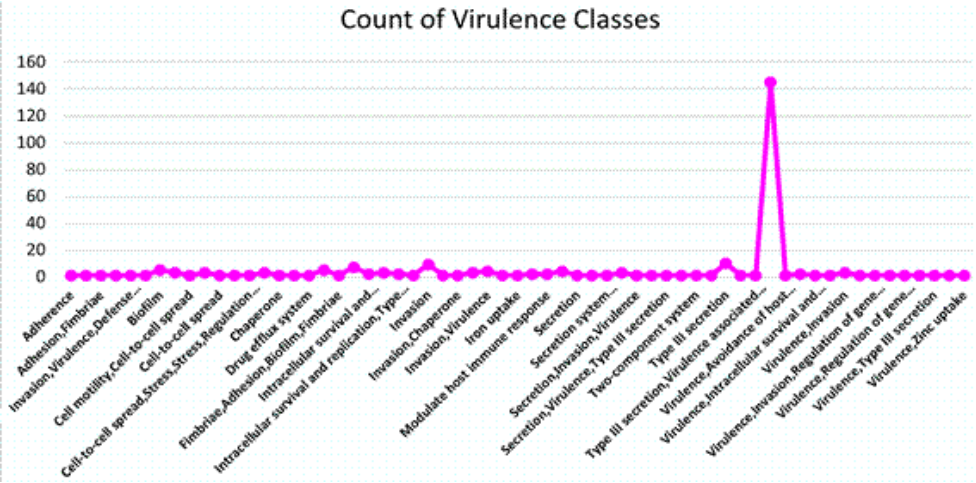

Fig. 2B: Graph representing Virulence classes of genes on the basis of their function and peak depicting most of the genes involved in Virulence associated with Type3 secretion.

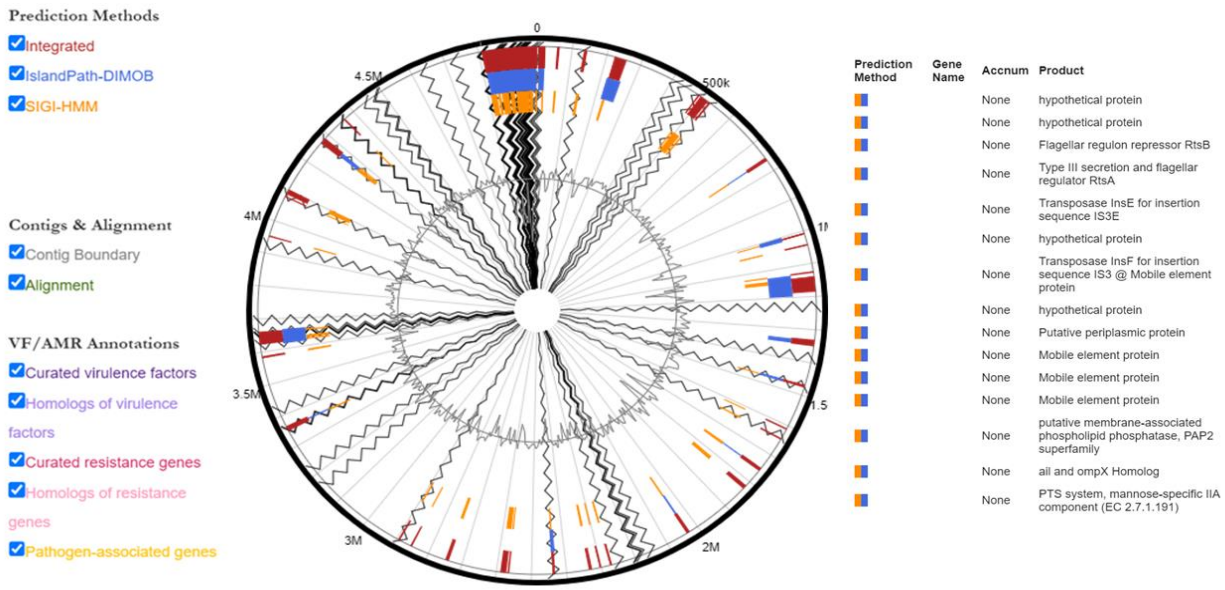

Fig. 2C: Islandviewer4 represents the Virulence (VRGs) and Antimicrobial Resistance genes (ARGs) in the isolated *S. enterica* genome. Color blocks showing the prediction methods, maroon color Integrated, blue color Islandpath-DIMOB, orange color SIGI-HMM. Grey color representing the contig boundries.

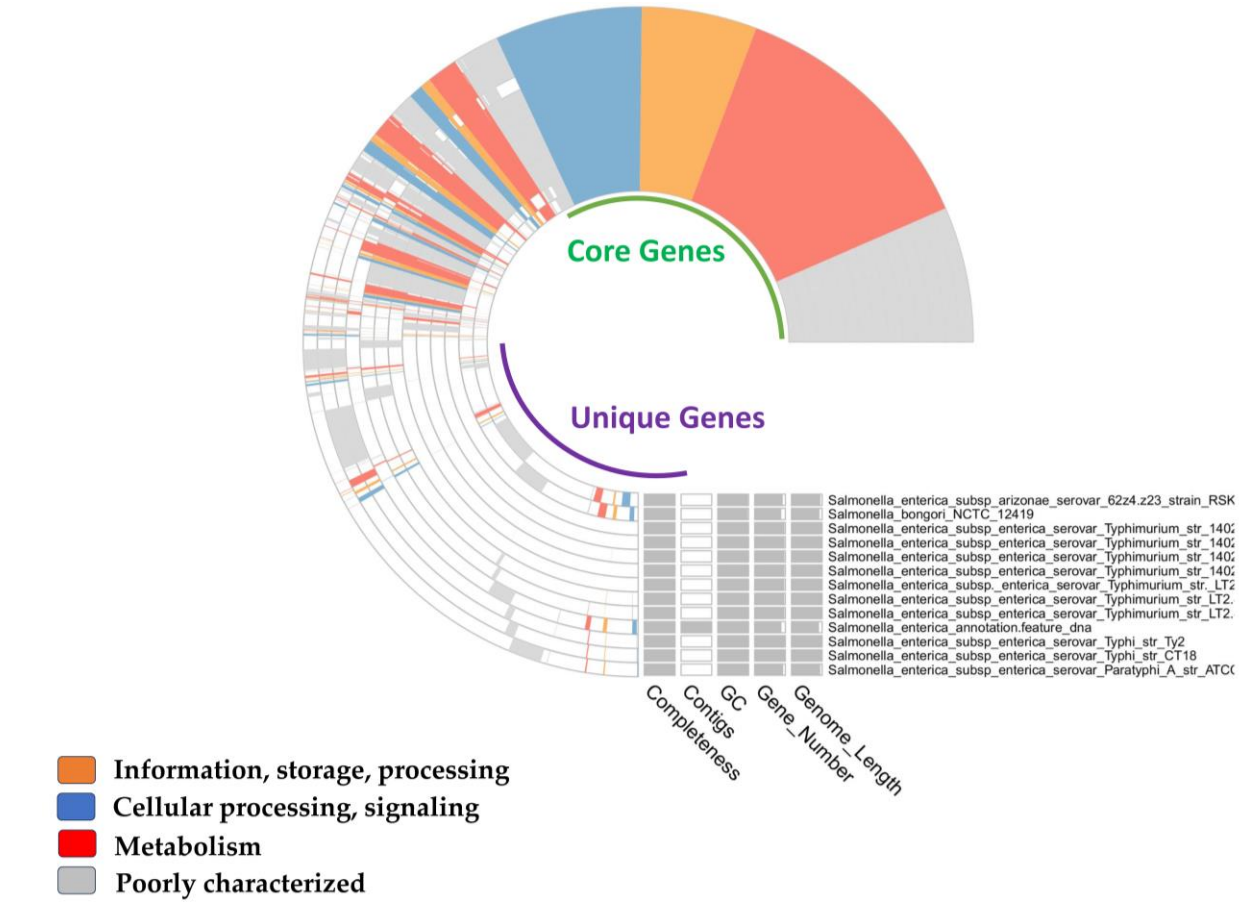

Fig. 3A: Illustrates the Core genes and Unique genes, Gene length, Gene Number, G+C content, Contigs and the completeness of the Genomes.

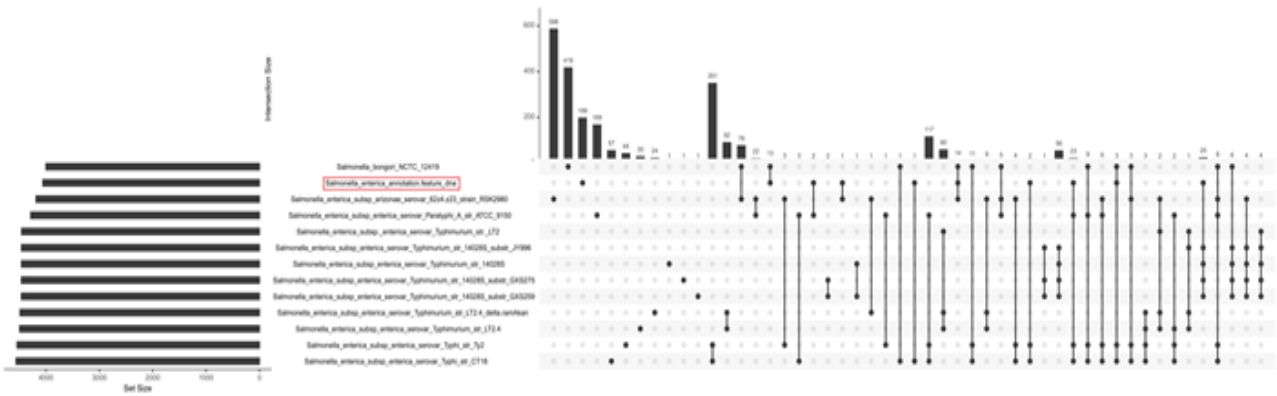

Fig. 3B: Upset Cluster showing the unique genes of each *Salmonella* strain, depicting Isolated *S.enterica* having 199 unique genes.

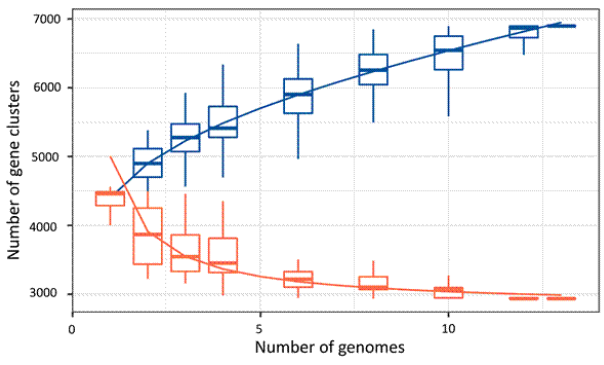

Fig. 3C: Graph representing number of Gene Clusters in 13 *S.enterica* strains, number of Pan Gene clusters in blue and number of Core Gene clusters in orange.

DISCUSSION

Nontyphoidal *S. enterica* is a significant cause of foodborne diseases and possesses the ability to transfer its virulence to other members of the pathogenic family, posing a considerable public health concern in recent years (Zhang *et al.*, 2022). Consequently, it is essential to learn the genetic characteristics of *S. enterica*, including its evolutionary relationships, comparative genomics and virulence factors involved in bacterial pathogenicity. To the best of our knowledge, this is the first report on genomic landscapes by WGS of *S. enterica* isolated from poultry and its related environment in Faisalabad city, Pakistan.

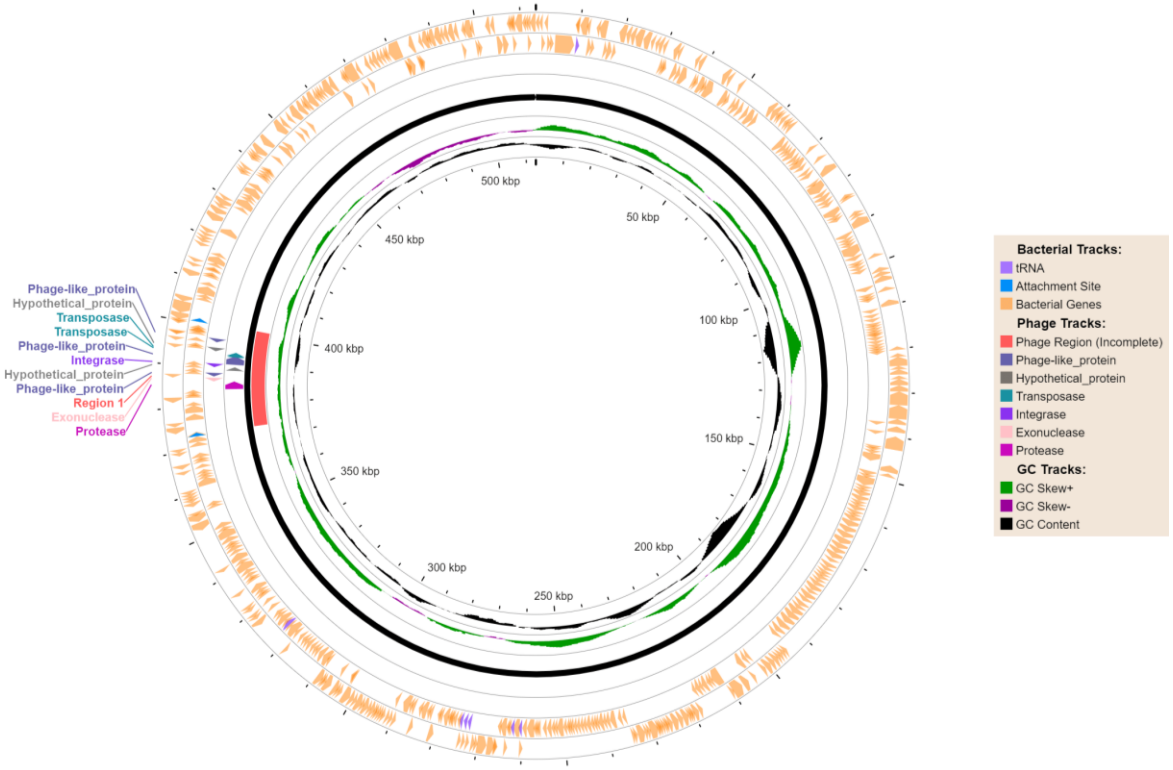

Fig. 4A: Circular genome showing the incomplete integration of lambda phage.

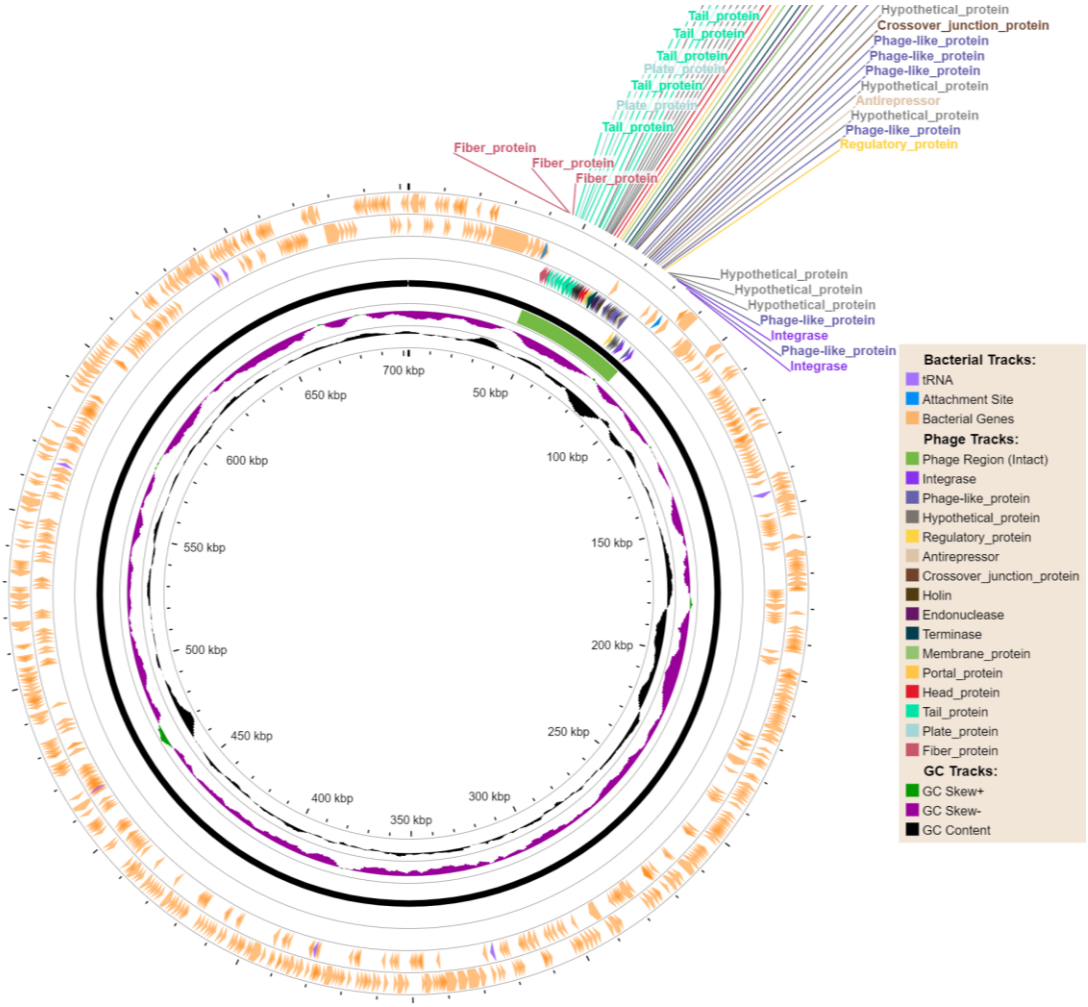

Fig. 4B: Circular genome representing the intact *Sal3* phage.

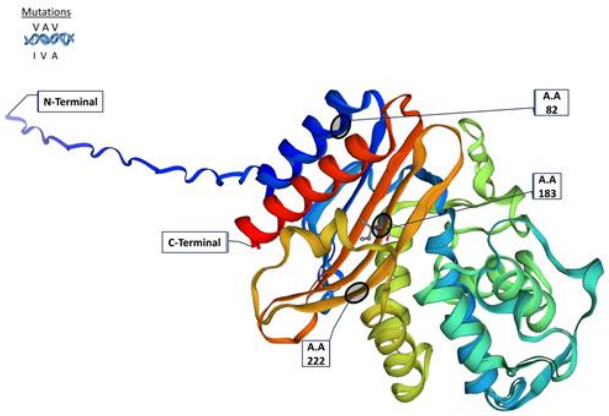

Fig. 5: Point mutations in 3D structure of *bla-TEM* 176, antibiotic resistant gene (ARG).

Multiple bioinformatics tools were employed to analyze the WGS of *S. enterica* isolated from poultry and its environment. The WGS of the isolate facilitated wgMLST and phylogenetic analyses, wgMLST indicating that these strains have the same origin to the pathogenic family of *S. enterica*. The phylogenetic analysis revealed the differences in the isolated strain of *Salmonella* with the reported strains in NCBI suggesting that this *Salmonella* strain may cause symptomatic salmonellosis. Phylogenetic analysis and clustering via wgMLST indicated that the isolated strain from the poultry environment has genetic relevance to previously reported strains (Stevens *et al.*, 2022).

The presence of plasmids in *Salmonella* is very common and they have difference in their gene content and number of transfer genes but their tracking is important because some of them contain AR genes as beta lactamases in plasmids (Carattoli *et al.*, 2021). The isolated genome of *S. enterica* revealed the presence of four plasmids in it which are conjugative, incompatible and mobilizable plasmids like *IncI2*, *IncX* and *IncQ1*, respectively. These plasmids are associated with resistance to beta-lactams, aminoglycosides, quinolones, *tetA* and other AR genes and may involve in the dissemination of these genes. Point mutation is also involved in the evolution of bacterial pathogens and it is reported that *S. enterica* is less susceptible to changes in genome (Zhou *et al.*, 2018). The presence of plasmids within the whole genome sequence demonstrated high resistance of *S. enterica* to antimicrobials such as carbapenems and aminoglycosides (Fuga *et al.*, 2021), underscoring the virulence of this Gram-negative bacterium. These plasmids are common in the *Enterobacteriaceae* family, including *Escherichia coli*, *Salmonella*, and *Klebsiella pneumoniae*, and they carry numerous ARGs that enhance pathogenicity and biofilm formation in the pathogen (Cai *et al.*, 2021). The isolated genome of *S. enterica* from poultry environment showed evolutionary behavior as a point mutation it became resistant to ciprofloxacin. The MDR strain of *S. enterica* explains the indiscriminate use of antibiotics in poultry, which is a significant global concern.

The identification of plasmids *IncQ1*, *IncX4*, *IncX1* and *IncI2* in the analyzed genome of *Salmonella enterica* showing the presence of mobile colistin-resistance (*mcr)* genes in the WGS. It is already found that *mcr* genes were localized on IncX4, IncHI2 and IncI2 plasmids as *mcr* 1.1 genes were present on *IncX4* plasmid (Sia *et al.*, 2020). One of the identified plasmids *IncX4* having epidemiological importance as it is self-transferring plasmid among multiple species of *Enterobacteriaceae.* The presence of *mcr* genes in *Salmonella enterica* genomes showing leading cause of morbidity in the world (Christenson, 2013). In our study the presence of identified plasmids in the isolate genome of *Salmonella enterica* serovar Typhimurium from poultry-environment showing the circulation of resistance genes in various subspecies of *Salmonella*.

Colistin has been broadly used as the last therapeutic option for the treatment of enteric diseases and growth promoting purposes in poultry. The continued use of colistin is the main root of emergence, transmission, and spread of resistance genes. The presence of *mcr* genes in whole genome sequences of the isolated *Salmonella enterica* from poultry and its related environment exhibiting the spread of resistance in foodborne strains. It is described that *mcr* induces resistance by encoding an enzyme, phosphoethanolamine transferase, involved in the modification of lipid-A compound of lipopolysaccharides (Kieffer *et al.*, 2019; Rau *et al.*, 2020).

Salmonella pathogenicity islands (SPIs) are specific area on the bacterial chromosome that contains various virulence factor involved in adhesion, invasion and toxicity. A total of twenty-three SPIs are present in different species of *Salmonella* from which two SPIs (SPI 1& 2) are known as molecular syringe because of the Type III secretion system (TTSS). The pathogen survived and proliferated inside the host cell with the help of effector proteins secreted by this TTSS (Coburn *et al.*, 2007) . The isolated genome of *Salmonella enterica* representing SPI 1 to 10 having multiple virulence factors involved in pathogenicity of *Salmonella* such as *invA, sipA, sipC* genes involved in the formation of TTSS which involved in the pathogenicity.

Genetic determinants known as virulence factors are distributed across various pathogenicity islands (SPIs), which are responsible for systematic transmission leading to severe infections (Kombade & Kaur, 2021). The virulence genes identified in the isolated whole genome range from 150 to 200 and include genes such as *invA*, *sipC*, *hilC*, *hilA*, *ssaK*, *fimA*, and *csgA*. These genes are involved in invasion via the TTSS, fimbriae formation, and biofilm development of the pathogenic bacteria. The presence of the T3SS and other virulence factors indicates the pathogen's ability to colonize the liver of the host that can potentially lead to serious illness (Veeraraghavan *et al.*, 2019). During the early phase of infection, the invasion of intestinal epithelial cells and M cells in the gut lumen activates proinflammatory responses mediated by the TTSS-1 cluster. In the late phase of infection, intracellular survival and replication within host phagocytes are associated with the TTSS-2 (Bao *et al.*, 2020). Both the virulence factors and resistance genes in the genome of *S. enterica* isolated from the poultry-environment showing the critical hygienic poultry conditions.

Gene enrichment analysis elucidated the infection pathway of *S. enterica* highlighting the export of virulent proteins into host cells, as well as the bacterial chemotaxis

pathway, which reflects the motility of the bacterial strain with gene mapping of known annotations. The infection pathway of isolated *S. enterica* genome showed the involvement of 28 virulence genes play potentially important role in the cause of disease. *Salmonella* has developed multiple strategies to invade and establish a systemic infection in various cell types, including epithelial cells, macrophages, dendritic cells, and M cells during the infection process. There are several routes for the dissemination of *S. enterica*, enabling the establishment of a persistent infection in the host (Li, 2022). Genome enrichment analysis and infection pathway mapping using the KEGG database reveal host-pathogen interactions and enhance our understanding of the pathogenesis caused by the isolated genome of *S. enterica*.

There were abundant prophages, with roughly 9,000 phage types, on *Salmonella* genomes which may harbor virulence genes (VRGs) and antibiotic resistance genes (ARGs) that encode proteins involved in bacterial pathogenesis. The genome of *S. enterica* had two phage genomes, *Sal3* phage and lambda phage, which are integrated (both "intact" and "incomplete") into its genome and contributed significantly to genetic diversification of *S. enterica* strain. These intact or incomplete genomes of phages may results in effector proteins which enhance the virulence of the host cell such as superoxide di-mutase, *sodC*, encoded by lysogenic phages involve to the establishment of *Salmonella* cells into the macrophage (Wahl *et al.*, 2019).

In this study, we identified mutations within the isolated genome of *S. enterica* which may result ARGs, as well as regulatory and virulence genes. The protein structures of selected genes were predicted and further analyzed for mutations by sequence alignment showed the effect on the structure of protein as well as its function. For instance, the *bla-TEM176* antimicrobial resistance gene in the isolated genome of *S. enterica* exhibited point mutations occurring in the alpha helices and beta chains of the protein structure. This indicated that because of these mutations the isolated strain of *S. enterica* may be susceptible to antibiotics than the other resistant strains. It is also reported that tat proteins involved in Type-III secretion of *Salmonella* while mutations in these proteins make them susceptible to antimicrobials than wild type strains (Reynolds *et al.*, 2011; Bao *et al.*, 2020). These secondary structures are crucial for maintaining protein conformation and functionality. Furthermore, the C-terminal and N-terminal domains of the protein structure influence its structural and functional sites. Drug targets in the isolated genome of *S. enterica* identified and then reported targets in the genome were aligned with the protein sequences of the selected *sipA* virulence gene. The protein structure of *sipA* clearly indicates the location of drug binding site. These drug targets are valuable for designing therapies against multidrug-resistant *S. enterica* strains.

Conclusions: Taking together, complete genome analysis exposed the evolutionary relationship of isolated *S. enterica* showing resistance against colistin from poultry-environment interface, which is a serious one health concern. Circulating *S. enterica* displaying the genetic modifications may be a cause of severe health and economic consequences as it is important to ensure safe and hygienic management of the poultry environment due to the high demand of poultry meat and egg products.

Authors contribution: Conceptualization, SM, BA, and MHR; methodology, SM, BA and MK; software, MHR and validation, BA, MHR, formal analysis, SM, and BA; investigation, SM, BA and MK; resources, BA, MHR; data curation, SM, and BA; writing—original draft preparation, SM, BA, and MHR; writing—review and editing, MK; visualization, MK; supervision, BA, MHR and MK; project administration, BA; funding acquisition, BA and MK All authors have read and agreed to the published version of the manuscript.

REFERENCES

Ali S and Alsayeqh AF, 2022. Review of major meat-borne zoonotic bacterial pathogens. Front Pub Health 10:1045599.

Altaf Hussain M, Wang W, Sun C, *et al.,* 2020. Molecular characterization of pathogenic Salmonella spp from raw beef in Karachi, Pakistan. Antibiotics 9(2):73.

Bao H, Wang S, Zhao JH, *et al.*, 2020. Salmonella secretion systems: Differential roles in pathogen-host interactions. Microbiol Res 241: 126591.

Bertelli C, Laird M R, Williams K P, *et al.*, 2017. IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets. N A Res 45(W1):W30-W35.

Brettin T, Davis J J, Disz T, *et al.*, 2015. RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Scient Reports 5(1):1-6.

Bu D, Luo H, Huo P, *et al.*, 2021. KOBAS-i: intelligent prioritization and exploratory visualization of biological functions for gene enrichment analysis. N A Res 49(W1):W317-W325.

Cai W, Tang F, Jiang L, *et al.*, 2021. Histone-like nucleoid structuring protein modulates the fitness of tet (X4)-bearing IncX1 plasmids in gram-negative bacteria. Fin Mic 12:763288.

Carattoli A, Villa L, Fortini D, *et al.*, 2021. Contemporary IncI1 plasmids involved in the transmission and spread of antimicrobial resistance in Enterobacteriaceae. Plasmid 118:102392.

Carattoli A, Zankari E, García-Fernández A, *et al.*, 2014. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Anti A Chem 58(7):3895-3903.

Christenson J C. 2013. Salmonella infections. Pediat Rev 34(9):375-383.

Coburn B, Grassl G A, & Finlay B.,2007. Salmonella, the host and disease: a brief review. Immunol Cell Biol 85(2):112-118.

Davis J J, Wattam A R, Aziz R K, *et al.*, 2020. The PATRIC Bioinformatics Resource Center: expanding data and analysis capabilities. Nucl Acids Res 48(D1):D606-D612.

Fatima A, Saleem M, Nawaz S, *et al.*, 2023. Prevalence and antibiotics resistance status of Salmonella in raw meat consumed in various areas of Lahore, Pakistan. Scient Reports 13(1):22205.

Florensa A F, Kaas R S, Clausen P T L C, *et al.*, 2022. ResFinder–an open online resource for identification of antimicrobial resistance genes in next-generation sequencing data and prediction of phenotypes from genotypes. Mic Genomics 8(1):000748.

Fuga B, Cerdeira L, Moura Q, *et al.*, 2021. Genomic data reveals the emergence of an IncQ1 small plasmid carrying blaKPC-2 in Escherichia coli of the pandemic sequence type 648. JGlobal A R 25:8-13.

Grant J R, Enns E, Marinier E, *et al.*, 2023. Proksee: in-depth characterization and visualization of bacterial genomes. Nucl Acids Res 51(W1):W484-W492.

Gutierrez A, De J & Schneider KR, 2020. Prevalence, concentration, and antimicrobial resistance profiles of Salmonella isolated from Florida poultry litter. J Food Protec 83(12):2179-2186.

Humphries R, Bobenchik AM, Hindler JA, *et al.*, 2021. Overview of changes to the clinical and laboratory standards institute performance standards for antimicrobial susceptibility testing, M100. J Clinical Microbiol 59(12):10-128.

Jain P, Chowdhury G, Samajpati S, *et al.*, 2020. Characterization of non-typhoidal Salmonella isolates from children with acute gastroenteritis, Kolkata, India, during 2000–2016. Bra J Mic 51:613-627.

Khan MUZ, Humza M, Yang S, *et al.*, 2021. Evaluation and optimization of antibiotics resistance profile against *Clostridium perfringens* from buffalo and cattle in Pakistan. Antibiotics 10(1):59.

Kieffer N, Royer G, Decousser JW, *et al.*, 2019. mcr-9, an inducible gene encoding an acquired phosphoethanolamine transferase in Escherichia coli, and its origin. Antimic A Chemo 63(9): 10.1128/aac. 00965-00919.

Kim H, Park S, Lee T, *et al.*, 2006. Identification of Salmonella enterica serovar Typhimurium using specific PCR primers obtained by comparative genomics in Salmonella serovars. J Food Prod 69(7): 1653-1661.

Kombade S & Kaur N, 2021. Pathogenicity island in Salmonella. In *Salmonella spp.*-A Global Challenge. IntechOpen.

Kong-Ngoen T, Santajit S, Tunyong W, *et al.*, 2022. Antimicrobial resistance and virulence of non-typhoidal Salmonella from retail foods marketed in Bangkok, Thailand. Foods 11(5):661.

Lamichhane B, Mawad A M, Saleh M, *et al.*, 2024. Salmonellosis: An overview of epidemiology, pathogenesis, and innovative approaches to mitigate the antimicrobial resistant infections. Antibiotics 13(1):76.

Lazăr R, Boişteanu P, Lazăr M, *et al.*, 2019. Isolation and identification of Salmonella in a Lohmann Brown chicken lot, raised in a traditional system. Scientific Papers Animal Science Series: Lucrări Ştiinţifice - Seria Zootehnie, vol. 71.

Li Q, 2022. Mechanisms for the invasion and dissemination of Salmonella. Can J Infec Dis Med Mic 2022(1): 2655801.

Liu D, Zhang Y, Fan G, *et al.*, 2022. IPGA: A handy integrated prokaryotes genome and pan-genome analysis web service. iMeta 1(4):e55.

Rau R B, de Lima-Morales D, Wink P L, *et al.*, 2020. *Salmonella enterica* mcr-1 positive from food in Brazil: detection and characterization. FB Pathog Dis 17(3):202-208.

Reynolds MM, Bogomolnaya L, Guo J, *et al.*, 2011. Abrogation of the twin arginine transport system in Salmonella enterica serovar Typhimurium leads to colonization defects during infection. PLoS One 6(1):e15800.

Rosignoli S and Paiardini A, 2022. Boosting the full potential of PyMOL with structural biology plugins. Biomol 12(12):1764.

Sedrakyan A, Ktsoyan Z, Arakelova K, *et al.*, 2022. Molecular epidemiology and virulence of non-Typhoidal Salmonella in Armenia. Inter J Mol Sci 23(16):9330.

Shakir MZ, Rizvi F, Javed M T, *et al.*, 2021. Seroprevalence and pathological studies of Salmonella infection in commercial white layer birds. Mic Pathog 159:105146.

Sia CM, Greig DR, Day M, *et al.*, 2020. The characterization of mobile colistin resistance (mcr) genes among 33 000 *Salmonella enterica* genomes from routine public health surveillance in England. Microb Genom 6(2):e000331.

Stevens EL, Carleton HA, Beal J, *et al.*, 2022. Use of whole genome sequencing by the federal interagency collaboration for genomics for food and feed safety in the United States. J Food Pro 85(5): 755-772.

Szklarczyk D, Gable A L, Lyon D, *et al.*, 2019. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucl Acids Res 47(D1):D607-D613.

Vázquez X, García V, Fernández J, *et al.*, 2022. Colistin resistance in monophasic isolates of Salmonella enterica ST34 collected from meat-derived products in Spain, with or without CMY-2 co-production. F in Mic 12:735364.

Veeraraghavan B, Jacob JJ, Prakash JAJ, *et al.*, 2019. Extensive drug resistant Salmonella enterica serovar senftenberg carrying bla NDM encoding plasmid p5558 (IncA/C) from India. Path Glo Health 113(1):20-26.

Wahl A, Battesti A & Ansaldi M, 2019. Prophages in Salmonella enterica: a driving force in reshaping the genome and physiology of their bacterial host? Mol Mic 111(2):303-316.

Wang M, Qazi I H, Wang L, *et al.*, 2020. *Salmonella* virulence and immune escape. Microorganisms 8(3):407.

Wang R H, Yang S, Liu Z, *et al.*, 2024. PhageScope: a well-annotated bacteriophage database with automatic analyses and visualizations. Nucl Acids Res 52(D1): D756-D761.

Waterhouse A, Bertoni M, Bienert S, *et al.*, 2018. SWISS-MODEL: homology modelling of protein structures and complexes. Nucl Acids Res 46(W1): W296-W303.

Wattam AR, Davis JJ, Assaf R, *et al.*, 2017. Improvements to PATRIC, the all-bacterial bioinformatics database and analysis resource center. Nucl Acid Res 45(D1): D535-D542.

Wishart DS, Han S, Saha S, *et al.*, 2023. PHASTEST: faster than PHASTER, better than PHAST. Nucl Acid Res 51(W1): W443-W450.

Yang Z, 2019. Optimised protocol of QIAamp® DNA mini Kit for bacteria genomic DNA extraction from both pure and mixture sample. Res Square. DOI: 10.21203/rs.2.17049/v1.

Yousafzai H, Rind R, Khan M, *et al.*, 2019. Microbiological contamination of cattle and buffalo meat in Peshawar. Pak J Anim Health Prod 7(1):11-16.

Zankari E, Allesøe R, Joensen K G, *et al.*, 2017. PointFinder: a novel web tool for WGS-based detection of antimicrobial resistance associated with chromosomal point mutations in bacterial pathogens. J AM Chemo 72(10):2764-2768.

Zhang J, Liu J, Chen C, *et al.*, 2022. Resistance and pathogenicity of Salmonella Thompson isolated from incubation end of a poultry farm. Vet Sci 9(7):349.

Zhou Z, Lundstrøm I, Tran-Dien A,*et al.*, 2018. Pan-genome analysis of ancient and modern *Salmonella enterica* demonstrates genomic stability of the invasive para C lineage for millennia. Curr Biol 28(15):2420-2428. e2410.