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Original Research Article

Exploring the traits, genetic variability and antibiotic resistance of *salmonella* serovars in poultry farming in Pakistan

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ABSTRACT

Background: Salmonellosis in poultry have been reported worldwide as the major cause of gastrointestinal foodborne illness and unembellished losses to the poultry industry. The current study aimed to isolate, purify, and characterize the *Salmonella* species in suspected chickens during natural outbreaks in commercial poultry farms in Pakistan.

Materials and Methods: A total of 50 dead birds were randomly selected and examined for postmortem from each of the 10 farms located in different areas of Punjab and Sind provinces of Pakistan. Liver and cloacal swab samples from each of the birds showing typical lesions were collected and inoculated on Salmonella-shigella agar (SSA), Xylose-Lysine deoxycholate agar (XLD) and CHROM agar (CA). The DNA of the isolates was extracted and amplified using an flhB gene specifically designed primer for Salmonella. The gene sequences of the different isolates were analyzed using the NCBI portal and a phylogenetic relationship was established (DNA-Star version 2.0).

Results: Small black center colonies with translucent margins appeared after 24 hours of incubation at 37°C, showing a 379bp PCR product. The validated sequences were deposited in the NCBI database with accession numbers PP393497 through PP726904. Gene sequence analysis revealed two different serovars of Salmonella species *Sal. enteritidis* (13%) and *Sal. typhimurium* (80%) while the remaining isolates did not predict any similarity to the NCBI database.

Conclusion: The isolates showing more than 99% similarity to accession numbers are CP011365, CP129209, CP011365, CP129206, CP011365, CP011365, CP011365, CP129206, CP011942, CP129209, CP129206, CP129209, LR792395and CP100724. Finally, most gram-negative bacteria are sensitive erythromycin, and tetracycline, and they also showed resistant to ofloxacin and gentamycin.

1. Introduction

Liver abnormality syndrome is a common problem in most of the commercial poultry farms worldwide. It is either caused by bacteria such as *Salmonella* species etc., or viral diseases such as Fowl adeno virus, Chicken anemia virus, avian herpes virus, etc., or nutritional problems such as feed toxicity and metabolic disorders like fatty liver hemorrhagic syndrome. Salmonellosis is one of the contagious bacterial diseases of domestic and wild avian species. *Salmonella* is gram-negative bacilli, capsular, non-spore former, motile bacteria belonging to the family Enterobacteriaceae are aerobe, oxidase-positive. The size of the bacteria is relatively small and most of the motile

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bacteria possess peritrichous flagella for tumble movement. It grows on Salmonella-shigella agar (SSA), MacConkey agar (MA) and Xylose-Lysine deoxycholate agar (XLD) and CHROM agar (CA). It ferments glucose, fructose, lactose, mannose, sucrose and galactose.[1]

The organism is divided into serotypes based on capsular (K), flagellar (H) and somatic (O) antigens. There are more than 2600 serovars reported all over the world in published reports[2] [2] but the common serovars associated with the disease are *Sal. enteritidis*, *Sal. Typhimurium*, *Sal. pullorum* and *Sal. gallinarum* when characterized through polymerase chain reaction.[3]

The symptoms vary from flock to flock but hurdling, off-feed, poor growth, drop in egg production, labored breathing, shivering, in coordination and whitish-colored diarrhea were observed commonly in every flock. In chronic form, the infection localizes in the oviduct, kidneys and even ureters leading to the source of vertical transmission to hatch chicks. On postmortem, the liver usually found enlarged and fragile and displayed severe congestion. The spleen usually swollen and nephritis and enteritis were evident in most of the cases. It is also reported in previously published research that pathogenic serovars of Salmonella from the contaminated hatching eggs disseminate vertically and proliferate in the chicks resulting in severe consequences in terms of morbidity, mortality and poor growth. It remains viable for more than nine weeks in the oviduct and liver of carrier birds.[4] The Salmonellosis outbreaks suddenly start with a drop in feed consumption followed by a descent in egg production. The disease evaluation markers such as fertility and hatchability begin to reduce with the onset of diarrhea. It has been revealed in many previously published reports that *Salmonella* serovars enterica can infect eggs during the developmental phase within the oviduct and is lingered as the source of vertical transmission.[5]

Extensive serovars of *Salmonella* can infect poultry but one serovar may persist in the population for a long time until substituted by other serovars the most common prominent serovars are *Sal. typhimurium* and *Sal. enteritidis*.[6] Salmonella has more reported serovars making it difficult to identify and classify for study of its surveillance. The conventional techniques to identify Salmonella are time-consuming and do not provide complete information regarding their genotype impediments and antibiotic resistance potential. Type-specific PCR-based techniques are encouraging in their use in Salmonellosis diagnosis due to their great specificity and sensitivity potential in exactly marking the target.[7,8]

Salmonellosis is extensively involved in liver abnormality syndrome of commercial layers and broiler breeders worldwide, particularly in countries with hot humid environments. However, information regarding the molecular characterization of indigenous isolates of *Salmonella* species in Pakistan is scanty. Therefore, this study is designed to isolate *Salmonella* from the broilers and layer flocks showing liver abnormality syndrome and their characterization through conventional and molecular techniques. The present study assists poultry researchers by using specific strains in immunization schemes to eliminate the *Salmonella* species from fertile hatching and broiler chickens.

2. Material and Methods

2.1. Sample collection and processing

Samples were collected from the suspected poultry flocks showcasing the salmonellosis-specific clinical signs including dullness, severe depression, anorexia, stood motionless about with head sunk into the chest and with both eyes closed ruffled feather and white or greenish diarrhea. The pathognomic lesions marked the congestion, hemorrhages, greyish necrotic foci and reddish hemorrhagic foci, perihepatitis, discoloration and enlargement of liver in salmonellosis suspected birds as showed in (Figure 1).[9] Upon postmortem, liver and cloaccal samples were collected from 50 infected birds from different areas of Punjab, Pakistan in sterile zipper bags and transferred to the laboratory for further processing in the ice box.

In the laboratory, the loopful of liver and cloacal swab samples were cultured separately on multiple selective media plates such as Salmonella shigella agar (SSA), MacConkey agar (MA), Xylose-Lysine deoxycholate agar XLD agar and CHROM agar (Merk-Germany).[10] All the plates were incubated at 37°C for 48 hours. A loop full of suspected colonies was further processed for a thin smear on the glass slide and the procedure finished with dry heat fixation. Gram staining was performed it separated the bacteria into gram positive and gram negative bacteria and the stained slide was observed under the compound microscope through a 100X oil immersion lens. Assumed *Salmonella* colonies were picked up, and preserved into semi-solid agar as stock medium and into slant agar for further biochemical, serological identification and molecular characterization.

2.2. Biochemical analysis

Moreover, the morphologically confirmed isolates were further characterized by performing the biochemical tests of the organism. The analytical profile index of the isolated organism was established through RapID One Pannel (Remel-Thermo Fisher Scientific, USA) following instructions of the manufacturer.[11,12]

2.3. Serological identification

Serogrouping of identified bacterial isolates was performed according to the Kauffmann–White method.[13]

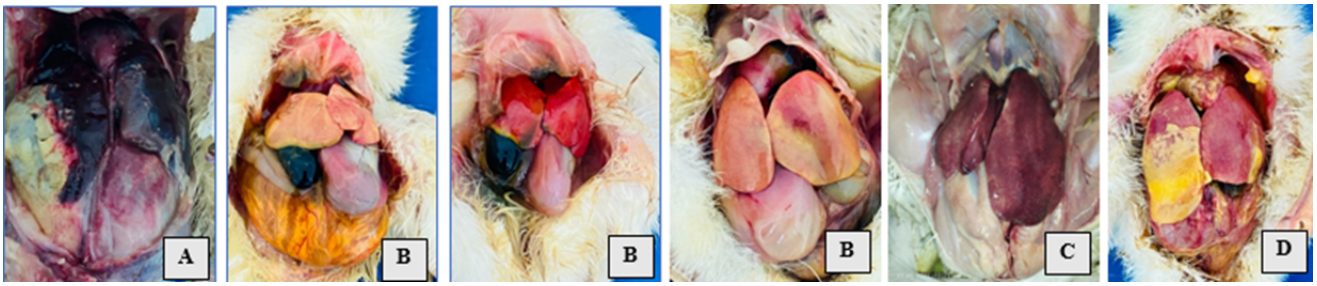

Figure 1: Salmonellosis suspected casesin poultry; **A:** Bird affected withfowl typhoid showing bronze discoloration of liver; **B:** Chick infected with salmonellosis showing bulging necrotic focion liver; **C:** Fowl affected withsalmonellosis showing haemorrhagic foci on liver; D: Bird diseased with salmonellosis showing small elevated greyishwhite nodular lesions on ventricular region of heart.

2.4. Molecular characterization

All phenotypically, serologically and biochemically confirmed isolates were further confirmed by targeting the flhb gene of *Salmonella* using a polymerase chain reaction. The nucleic acid of each isolate ware extracted by using a DNA extraction kit (Qiagen- France) according to the manufacturer instructions.

The 379bp-sized forward primer: ATCGCTGACTTATGCAATCG and reverse primer: CGGGTTGCGTTATAGGTCTG were synthesized by Genome-Malaysia. The polymerase Chain Reaction (PCR) run recipe was standardized following the standard protocol with little modification using the thermocycler veritii by Applied Bio System-USA.[11] In a reaction mixture, 12μl of the Master mix, 7μl of nuclease-free water, 1μl of each primer and 4μl of DNA template to make the total volume 25μl were used. PCR-cycling was performed with initial denaturation of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min. The final extension was done at 72°C for 5 min. Finally, a presumed DNA amplicon size of 379 bp was observed in a horizontal gel electrophoresis system containing 1% agarose (Thermo Fisher-USA) gel stained with 1.5% ethidium bromide (Calbiochem-India) in 1X TAE buffer (Applichem- Germany) and analyzed under an ultraviolet trans illuminator (Vilber Lourmat-France).

2.5. Phylogenetic analysis

The PCR product was sequenced by APICAL SCIENTIFIC SDN. BHD., Malaysia followed by a Basic Local Alignment Search Tool (BLAST) assessment to compare it with the submitted data in NCBI. Moreover, DNA-STAR version 2 software was employed for constructing a maximum likelihood tree for phylogenetic analysis of indigenous Salmonella isolates.

2.6. Antibiotic sensitivity test

All molecular confirmed isolates were examined for their antibiotic sensitivity test by disc diffusion method.[14] Antibiotic discs infused with amoxicillin (AX, 10μg), florofenicol (FFC, 30μg), fosfomycin (FF, 200μg), enrofloxacin (ENR, 10μg), amoxicillin (AX, 25μg), doxycycline (DO, 30μg), neomycin (N, 30 μg), Ofloxacin (OFX, 5μg), Tylosin (TY, 30μg), Pefloxacin (PEF, 5μg), Norfloxacin (NOR, 10μg), colistin (CT, 10μg), furaxolidine (FX, 100μg), gentamycin (CN, 10μg), lincomycin, (L-2, 2μg) (HiMedia, India) were used. The diameter of the zones of complete inhibition was measured with digital caliper (Fisherscientific-USA) and compared with the zone size interpretation chart provided by the supplier and was graded as sensitive, intermediate and resistant. The multiple antibiotic resistance (MAR) index was also calculated for all Salmonella isolates following the protocol described by Krumperman, by applying formula a/b where “a” is the number of antibiotics to which an isolate was resistant and “b” is the number of antibiotics to which the isolates were exposed.[13]

3. Results

Colony morphology, microscopy, Biochemical analysis and molecular characterization results revealed a total of 15 probable *Salmonella* isolates. Each of the isolates showed black, viscous, mucoid, translucent and non-hemolytic colonies on Salmonella shigella agar, red colonies on Xylose-Lysine deoxycholate agar and magenta on chrome agar after 24 hours of incubation at 37^{o}C as depicted in (Figure 2). The bacteria were found in the Salmonella genus are gram-negative with peritrichous flagella. Each of the isolates was pathogenic and reactivated by inoculating into selective media incubated at 37°C for 24 hours. A thin glass slide impression when stained with grams of reagent showed pinkish rods in microscopy. The isolates were positive to oxidase, H_2S production, catalase, urease, indole production, nitrate reduction, citrate utilization, gelatin liquefaction, glucose, arabinose, maltose, lactose, dulcitol,

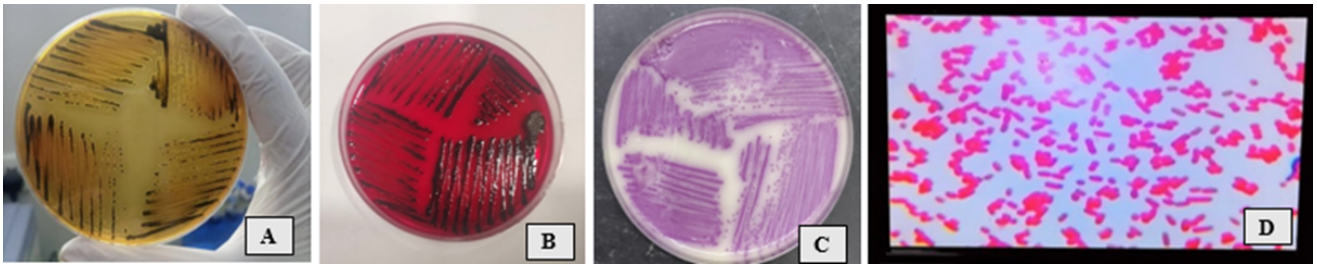

Figure 2: Morphological appearance on selected culture media; **A:** Black colonies on SSA; **B:** Blackcolonies on XLD; **C:** Magenta colonies on chrome agar; **D:** Results of gramstaining showed gram –ve rods

Table 1: Biochemical profile of salmonella by rapid one kit.

Bac.	URE	ADH	ODC	LDC	TET	LIP	KSF	KSF	GUR	OPNG	βGLU	βXYL	NAG	MAL	PRO	PRO	GGT	PYR	PYR	ADONIND
Sl.	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Abbreviation: Urea(URE), Arginine(ADH), Ornithine(ODC), Lysine(LDC), Aliphatic thiol(TET), Fatty acid ester(LIP), Sugar aldehyde(KSF), Sorbitol(SBL),p-Nitrophenyl-β, D-glucuronide(GUR), o-Nitrophenyl-β, D-galactoside(OPNG), p-Nitrophenyl-β, D-glucoside(βGLU), p-Nitrophenyl-β, D-xyloside (βXYL), p-Nitrophenyl-N-acetyl-β, D- glucosaminide (NAG), Malonate(MAL), Proline-β-naphthylamide(PRO), γ-Glutamyl- β-naphthylamide(GGT), Pyrrolidonyl- β- naphthylamide (PYR), Adonitol (ADON), Tryptophane(IND)

inositol and sucrose fermentation as showed in (Table 1).

The extracted DNA of each bacterial isolate was successfully targeted by using the *flh*B primer gene and exhibited the desirable amplicon of 379bp was declared as a *Salmonella* species showed in (Figure 3). The bacterial isolates from outbreaks of broilers and long-living birds showing abnormal liver syndrome belong to Serovars *Sal. enteritidis* (13%) and *Sal. typhimurium* (80%) while the remaining percentage account for the sequence that did not show any significant similarity to the NCBI database. *flh*B gene-based flagellar type *Sal. enteritidis* and *Sal. typhimurium* are declared as the primary cause of Salmonella infection in domestic and commercial poultry. Gene sequences of all the isolates were submitted to the NCBI gene bank with accession numbers PP393497, PP418878, PP393498, PP511204, PP511205, PP511532, PP511206, PP511207, PP713041, PP713042, PP537590, PP726903, PP726904 for OP13 to OP27 respectively except for OP22 and OP24 that are in the process of submission as illustrated in (Table 2).

The phylogenetic analysis based on multiple sequence alignment results showed isolates OP13 (PP393497), OP14 (PP418878), OP15 (PP393498), OP16 (PP511204), OP24 (in-process), OP25 (PP537590), OP26 (PP726903) and OP27 (PP726904) exhibited 99% similarity to the NCBI gene bank accession numbers CP011365, CP129209, CP011365, CP129206, CP129206, CP129209, LR792395 andCP100724 respectively revealing the slightest clade difference in the phylogenetic tree with the *Sal. typhimurium* except for OP27 that showed similarity to *Sal. enteritidis* as showed in Figure 4. Similarly, isolates OP17 (PP511205), OP18 (PP511532), OP19 (PP511206), OP20 (PP511207), OP21 (PP713041) and OP23 (PP713042) showed 100% similarity to the accession numbers CP011365, CP011365, CP011365, CP129206, CP011942, CP129209 respectively by the lowest clade difference with *Sal. typhimurium* established in the phylogenetic tree. However, OP22 did not show any significant similarity in the BLAST search as explained in (Table 2).

Note: The comparison of the nucleotide sequence of indigenous isolates with that of similar sequences submitted in NCBI via a BLAST search showed mutations at various positions in the nucleotide sequences of the study isolates. NCBI percentage nucleotide identity analysis of OP13 (PP393497), OP14 (PP418878), OP15 (PP393498), OP16 (PP511204), OP24 (In-process), OP25 (PP537590), OP26 (PP726903), and OP27 (PP726904) with CP011365.1, CP129209.1, CP011365.1, CP129206.1, CP129206.1, CP129209.1, LR792395.1, and CP100724.1 predicted the minimum nucleotide mutations at positions (15, 21, 22), (13, 15, 17, 21), (15, 21, 22), (14, 16, 20), (18, 30, 337), (27, 334), (20, 21), and (14, 20, 21) respectively with each having percentage identity of 99% with the subject sequence as showed in (Figures 5, 6 and 7).

The PCR confirmed Salmonella showed varying susceptibility pattern to 14 antimicrobial drugs as shown in table-3. The results revealed that the isolates are highly sensitive to fosfomycin (FF) 57%, furaxolidone (FX) 47%, doxycycline (DO) 42% followed by Colistin (CT), enrofloxacin (ENR) with percentage of 30% and 25%. It showed low susceptibility to pefloxacin (PEF) 7.5% whereas, the Salmonella was highly resistant to amoxicillin (AX), gentamycine (CN), tylosin (TY), florofenicol (FFC), neomycin (N) and ofloxacin (OFX) 0% as explained in (Table 3).

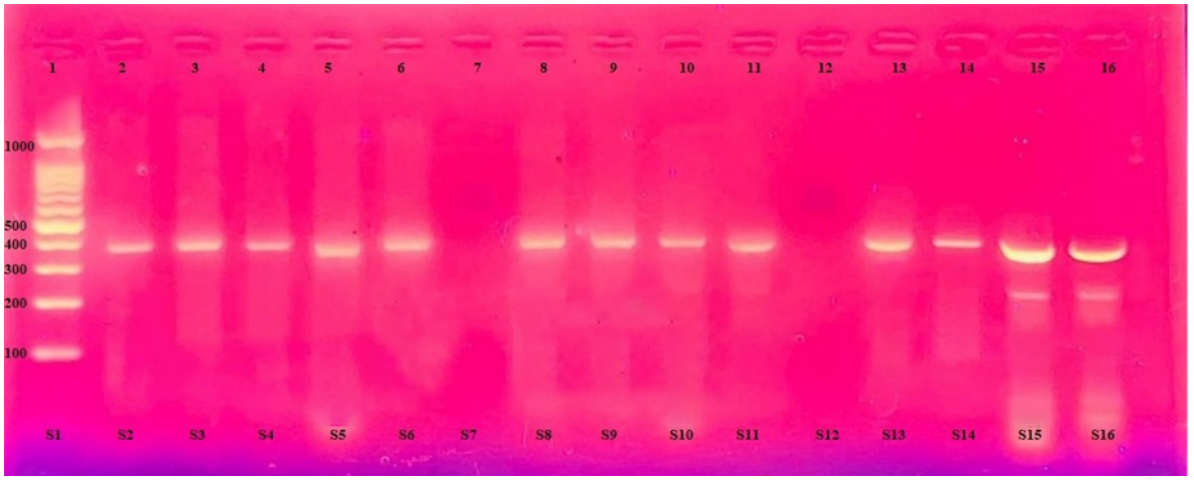

Figure 3: PCR for the detection of salmonella spp. The PCR amplifies the product of 379bp. Lane1: DNA marker (Trnas2K-China) Lane: 2-6,8-11 and 12-16 are DNA template of Salmonella isolates while, Lane: 7 and 12 are negative control.

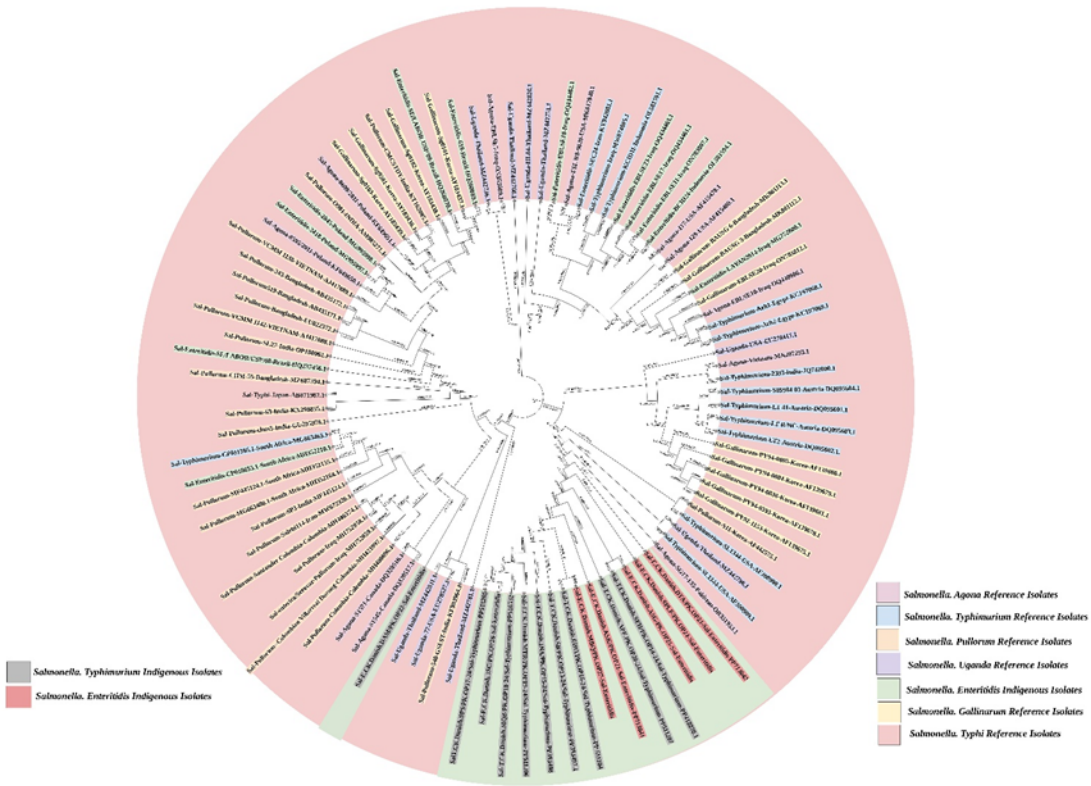

Figure 4: Phylogenetic analysis of flagellar flhB gene sequenced of Salmonella isolates aligned with DNA star Version 2. The region analyzed was a 379 base pair fragment atthe 3' of flhB gene. Branch length represents the predicted number ofsubstitution and are proportional to the difference between indigenous and reference isolates. The group of indigenous isolates selected for this studyare indicated on the tree as olive green shaded and the reference group shadedin peach color.

Table 2: Details history of PCR confirmed Indigenous Isolates of Salmonella spp. from Punjab, Pakistan sequenced in this study

S.No #	Title	Date	Location	Age/ Weight	Breed	Reported Mortality (%)	NCBI % Similarity	NCBI Blast A#	Strain	Serotype	NCBI OP A#
1.	SalT.CK.-Danish.-SPS/PK.OP13-24	06-01-24	Sheikhupura	20/1026	Breeder	19%	99%	CP011365.1	FORC_015	Sal subsp enterica serovar Typhimurium	PP393497
2.	SalT.CK.-Danish.-SEH/PK.OP14-24	19-01-24	Lahore	25/1500	Breeder	13%	99%	CP129209.1	ST_F0903R	Sal subsp enterica serovar Typhimurium	PP418878
3.	SalT.CK.-Danish.-INA/PK.OP15-24	12-01-24	Lahore	23/1480	Broiler	10%	99%	CP011365.1	FORC_015	Sal subsp enterica serovar Typhimurium	PP393498
4.	SalT.CK.-Danish.-EHO/PK.OP16-24	22-02-24	Lahore	22/1536	Broiler	12%	99%	CP129206	RM095	Sal subsp enterica serovar Typhimurium	PP511204
5.	SalT.CK.-Danish.-SPS/PK.OP17-24	24-02-24	Sahiwal	21/1609	Broiler	15%	100%	CP011365.1	FORC_015	Sal subsp enterica serovar Typhimurium	PP511205
6.	SalT.CK.-Danish.-MQB/PK.OP18-24	16-02-24	Bhera	25/1342	Breeder	20%	100%	CP011365.1	FORC_015	Sal subsp enterica serovar Typhimurium	PP511532
7.	SalE.CK.-Danish-AFM/PK.-OP19-24	19-02-24	Multan	26/1589	Broiler	13%	100%	CP011365.1	FORC_015	Sal subsp enterica serovar Enteritidis	PP511206
8.	SalT.CK.-Danish.-MTR/PK.OP20-24	20-02-24	Raiwind	33/2090	Broiler	19%	100%	CP129206.1	RM095	Sal subsp enterica serovar Typhimurium	PP511207
9.	SalT.CK.-Danish.-ASK/PK.OP21-24	05-03-24	Karachi	37/1882	Broiler	17%	100%	CP011942.1	ZC055	Sal subsp enterica serovar Enteritidis	PP713041
10.	SalT.CK.-Danish.-DAM/PK.OP22-24	03-03-24	Multan	35/1710	Broiler	11%	No Significant Similarity				
11.	SalT.CK.-Danish.DTA/PK.OP23-24	01-03-24	Okara	36/1900	Broiler	16%	100%	CP129209.1	H5_120Salm	Sal subsp enterica serovar Typhimurium	PP713042
12.	SalT.CK.-Danish.SP-LF/PK.OP24-24	02-03-24	Gujranwala	34/2190	Broiler	20%	99%	CP129206.1	HS_187Salm	Sal subsp enterica serovar Typhimurium	In-process
13.	SalT.CK.-Danish.-ASG/PK.OP25-24	04-03-24	Gujrat	35/1690	Broiler	14%	99%	CP129209.1	H5_120Salm	Sal subsp enterica serovar Typhimurium	PP537590
14.	SalT.CK.-Danish.-JSC/PK.OP26-24	22-01-24	Basipur	36/1986	Broiler	18%	99%	LR792395.1	CVM N38232	Sal subsp enterica serovar Typhimurium	PP726903
15.	SalE.CK.-Danish.-BCF/PK.OP27-24	24-03-24	Gujranwala	33/1745	Broiler	11%	99%	CP100724.1	R17.1476	Sal subsp enterica serovar Enteritidis	PP726904

Figure 5: Nucleotide mutation analysis of sequence flagellar flhB gene of Salmonella isolatesOP13- OP17

Table 3: Antibiogram of salmonellosis positive poultry flock

Antibiotics	**Concentration**	**Antibiotic Susceptibility Pattern of Isolated Salmonella Species**	
		Sensitive	**Resistant**
Florfenicol (FFC)	30ug	0%	100%
Fosfomycin (FF)	200ug	57%	43%
Enrofloxacin (ENR)	10ug	25%	75%
Amoxicillin (AX)	25ug	0%	100%
Doxycycline (DO)	30ug	42%	58%
Neomycin (N)	30ug	0%	100%
Ofloxacin (OFX)	5ug	0%	100%
Tylosin (TY)	30ug	0%	100%
Pefloxacin (PEF)	5ug	7.5%	92.5%
Norfloxacin (NOR)	10ug	15%	85%
Colistin (CT)	10ug	30%	70%
Furaxolidone (FX)	100ug	47%	53%
Gentamicin (CN)	10ug	0%	100%
Lincomycin (L-2)	2ug	0%	100%

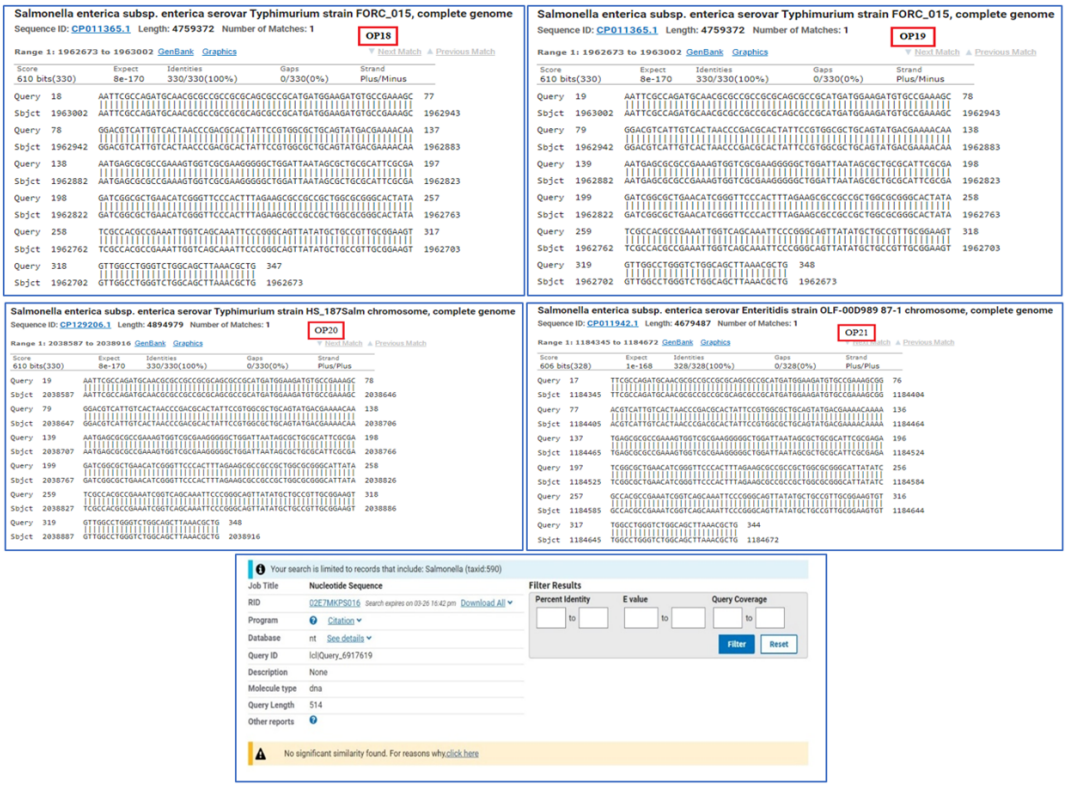

Figure 6: Nucleotide mutation analysis of sequence flagellar flhB gene of Salmonella isolates OP18- OP22

Figure 7: Nucleotide mutation analysis of sequence flagellar flhB gene of Salmonella isolatesOP23- OP27

4. Discussion

Cases of liver abnormality syndromes were observed in commercial broilers, layer, and broiler breeder flocks in various locations across Pakistan, including Sheikhupura, Lahore, Sahiwal, Bhera, Multan, Raiwind, Karachi, Multan, Okara, Gujranwala, Gujrat and Basipur. The farms reported a minimum mortality rate of 10%, up to 20% in some cases. The affected birds exhibited symptoms such as depression, anorexia, ruffled feathers, mucous discharge from the mouth and nares, laboured breathing. Additionally, the birds showed diarrhea and whitish-colored pasting at the vent. Postmortem examinations revealed petechial haemorrhages on serosal membranes, focal hepatic congestion and turbid fluid in the pericardial sac, hemorrhages in the sub-epicardium, and splenomegaly. It has been documented in various studies that Salmonella infection in poultry can lead to systemic infection with the involvement of multiple organs such as the liver, kidneys, and intestine.[15]

The bacterial isolates obtained from outbreaks of broilers and long-living birds showing abnormal liver syndrome have been identified as belonging to Serovars *Enteritidis* (13%) and *Typhimurium* (80%). In most salmonellosis outbreaks resulting from the consumption of poultry products, *Enteritidis* and *Typhimurium* serovars have been isolated.[16] Results showed that *Sal. enteritidis* was detected in 43% (6/14) and *Sal. typhimurium* in 36% (5/14) of the samples.[17] *Salmonella* infection of laying hens is dominated by Salmonella Enteritidis (SE) and ST is the principal cause of egg-associated salmonellosis outbreaks.[18]

The remaining percentage comprises sequences that did not exhibit significant similarity to the NCBI database. Based on the flhB gene, Enteritidis and Typhimurium flagellar types have been identified as the primary causes of *Salmonella* infection in domestic and commercial poultry. Accession numbers for the gene sequences of all the isolates have been submitted to the NCBI gene bank, ranging from PP393497 to PP726904 for OP13 to OP27, respectively.

In our phylogenetic analysis, we observed that the following isolates exhibited high similarity to specific gene bank accession numbers: OP13 (PP393497), OP14 (PP418878), OP15 (PP393498), OP16 (PP511204), OP24 (in-process), OP25 (PP537590), OP26 (PP726903), and OP27 (PP726904). These isolates displayed a 99% similarity to specific gene bank accession numbers, suggesting a slight clade difference in the phylogenetic tree with *Sal. typhimurium.* Notably, OP27 showed similarity to *Sal. enteritidis*. Furthermore, isolates OP17 (PP511205), OP18 (PP511532), OP19 (PP511206), OP20 (PP511207), OP21 (PP713041), and OP23 (PP713042) showed 100% similarity to specific gene bank accession numbers, indicating the lowest clade difference with *Sal. typhimurium* in the phylogenetic tree. However, the genes flhB and flhA encode proteins of 383 and 692 amino acids, with calculated molecular masses of 42,322 and 74,848 Daltons, respectively. These proteins are highly hydrophobic. FlhB exhibits substantial similarity to B. subtilis FlhB (38% identity) (5), and both of these proteins share homology with another family of virulence proteins, such as *Sal. flexneri* Spa4O (34) and *Sal. typhimurium* SpaS (10). Increasing evidence suggests that many flagellar proteins are highly similar to various virulence proteins required for the surface presentation of invasion antigen.[19]

The membrane protein FlhB plays a critical role in the flagellar secretion system by regulating protein export. The bacterial flagellum is a large and complex molecular machine with over 30 proteins organized into three primary substructures: the basal body, the hook, and the filament. Most flagellar proteins are located outside the cell and are transported across the cytoplasmic membrane by the flagellum-specific secretion apparatus. In the case of Salmonella enterica serovars Typhimurium (*Sal. typhimurium*), the flagellar secretion system, which is vital for bacterial pathogenicity, consists of six integral membrane proteins: FlhA, FlhB, FliO, FliP, FliQ, and FliR, along with three cytoplasmic proteins: FliH, FliI, and FliJ. Two proteins, the membrane protein FlhB and the hook-length control protein FliK, are essential and crucial for substrate switching. FlhB receives a signal from FliK to switch substrate specificity. If the fliK gene is deleted, the substrate switching does not occur, resulting in a very long hook, termed "polyhook," without any filament attached.[20]

In the context of Salmonella enterica serovar Typhimurium (ST), substrate switching refers to a critical process involving the Type Three Secretion System (T3SS), a sophisticated nanomachine complex found in several gram-negative bacteria, including *Sal. typhimurium*. This system enables bacteria to inject effector proteins directly into host cells, modulating various host cell responses, including cytoskeleton rearrangement, signal transduction, and cytokine production, ultimately increasing the bacterial pathogenicity. During infection, *Sal. tphimurium* activates two distinct T3SSs at different phases. The T3SS delivers effector proteins into host cells, where the order in which the proteins are injected matters. Early substrates are injected first, followed by middle and late substrates. Shifting from early to middle/late substrates is crucial for successful infection. The T3SS and needle complex in many bacterial pathogens of plants and animals have evolved a specialised protein-secretion system termed type III to deliver bacterial proteins into host cells.

The *Sal. typhimurium* pathogenicity island 1 encodes a system that helps the bacterium enter nonphagocytic cells by interfering with host cellular functions. The type III secretion system consists of a needle complex, which includes a base spanning both membranes and a needle-like projection extending outward from the bacterial surface. The assembly of the needle substructure requires the type

III secretion export apparatus, while the base assembly does not. The length of the needle segment is determined by the type III secretion-associated protein InvJ. Additionally, the base is composed of InvG, PrgH, and PrgK, while PrgI is the main component of the needle.[21] The rod assembly process involves the export of subunits of four proteins (FlgB, FlgC, FlgF, and FlgG) via the flagellum-specific pathway and their incorporation into the rod structure. This process requires the flhA and fliI genes, possibly because they encode part of the export apparatus. Once the rod assembly is complete, the FlgI and FlgH proteins assemble around the rod to form the P and L rings.[22]

5. Conclusion

In conclusion, the study identified and characterized Salmonella species in suspected chickens during natural outbreaks in commercial poultry farms in Pakistan. The study revealed the presence of two different serovars of Salmonella species, *Sal. enteritidis* and *Sal. typhimurium*, with most isolates belonging to the Typhimurium serovar. The findings also indicated that most gram-negative bacteria are sensitive to ofloxacin, erythromycin, and tetracycline, as well as to ofloxacin and gentamycin. The study highlighted the significant impact of Salmonella on commercial poultry farms, particularly in the context of liver abnormality syndrome in layers and broiler breeders. Identifying specific serovars and their antibiotic sensitivity patterns can contribute to developing targeted control and prevention strategies to mitigate the impact of *Salmonella* outbreaks in poultry farms. Furthermore, the study emphasized the importance of using molecular techniques such as PCR-based methods for accurate and timely identification of Salmonella serovars, especially in surveillance and control programs. Overall, the findings provide valuable insights for managing and controlling Salmonella-related issues in commercial poultry farming in Pakistan.

6. Conflict of interest

None.

7. Source of Funding

None.

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