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Full Length Article

Epidemiology of Indigenous Strains of Avian *Salmonella* in Commercial and Backyard Poultry in District Faisalabad, Pakistan

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Abstract

Avian salmonellosis is a disease of major concern for the health of poultry as well as humans and it causes great economic losses in terms of productivity in birds and cost of treatment. It is highly pathogenic for young birds; however adult birds remain lifelong carriers. In the present study, molecular epidemiology of avian salmonellosis was studied in commercial and backyard poultry through various diagnostic procedures to identify the avian salmonellosis. The correlation of associated risk factors was also studied in perspective to disease transmission. A combination of conventional and latest diagnostic methods *i.e.*, culture and recent techniques such as polymerase chain reaction and loop mediated isothermal amplification assay were used in this study. The *Salmonella* isolates resulted in straw colored or colorless colonies with black centers on *Salmonella* Shigella agar, greyish white colonies on blood agar and red colored colonies with black centers on Xylose Lysine Deoxycholate agar. A total of 47/376 samples were found positive, indicating 12.5% prevalence in the study area. Molecular confirmation revealed that four (04) different serovars of *Salmonella* are circulating in the study area. *Salmonella enteritidis* was the most prevalent serovar followed by *Salmonella gallinarum*, *Salmonella pullorum* and *Salmonella typhimurium*. Hence it was concluded that two zoonotic serovars *S. enteritidis* and *S. typhimurium* has been circulating in both types of poultry flocks including commercial and backyard.

Keywords: *Salmonella*; Molecular techniques; Zoonosis; Backyard; Commercial poultry

Introduction

Backyard poultry farming is very ancient in Indo-Pak region. This is the small-scale business that needs low input, however, natural hatching of eggs, poor production rate and absence of veterinary care services are the causes of major losses. About twenty to fifty birds of different ages and breeds are kept in backyard flock (Waheed *et al.* 2023; Zahid *et al.* 2024). In Pakistan, ~95.50 million birds are found in backyard system (GOP 2024) and most common breeds are Desi, Rhode Island Red, Aseel, Fayoumi, Naked Neck, Black Australorp and numerous crossbreeds (Zahid *et al.* 2024). It has been reported that backyard poultry is a source of *Salmonella* infection for humans, even if birds appear healthy and show no signs of the disease; still people can acquire *Salmonella* infection through environment or by touching live backyard chickens. In 2020, Center for Disease Control and Prevention (CDCP) and other public health authorities inspected the outbreaks of *Salmonella* in fifty states those were linked to contact with backyard flocks. The number of diseased cases reported this particular year was higher as compared to previous outbreaks linked to backyard poultry (Centers for Disease Control and Prevention 2020). According to the CDCP, 470 individuals from 48 states were infected with one of the outbreak strains of *Salmonella* in 2024. In a survey conducted by state and municipal public health officials, 217 (66%) of 330 individuals confirmed having contact with backyard chickens prior to being ill (Centers for Disease Control and Prevention 2024).

The genus *Salmonella* consists of gram-negative bacteria which are facultative anaerobes. These bacteria belong to Eneterobateriaceae family, motile, non-spore-forming and rod-shaped. The bacteria in this family are closely related to each other both genotypically and phenotypically as well as taxonomically. This genus contains two species *i.e.*, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies: *Enterica*, *Salamae*, *Arizonae*, *Diarizonae*, *Houtenae* and *Indica*. *S. pullorum* and *S.*

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sallinarum are typical causes of avian salmonellosis and prevalent around the globe. It is reported that many developed countries have eradicated these diseases from intensive farming units, but they are still causing heavy economic losses in developing countries. Both *S. pullorum* and *S. gallinarum* are non-motile in nature (Alzwghaibi *et al.* 2019; Shaji *et al.* 2023).

Many non-typhoidal *Salmonella* such as *S. enterica* serovar *enteritidis* and serovar *typhimurium* are responsible for causing disease in a variety of hosts such as bird, animals and humans (Shaji *et al.* 2023; Mahmood *et al.* 2025). These serovars are mostly linked with gastrointestinal infections in humans but other serotypes, for example *Salmonella infantis*, can also cause disease in humans and pose a public health risk (El-Fatah *et al.* 2020; Shaji *et al.* 2023). *Salmonella* is transmitted both vertically and horizontally in poultry birds and they can acquire infection from feces, also.

The clinical signs of avian salmonellosis are chalky white diarrhea, depression, anorexia, vent pasting, weight loss, growth retardation, huddling of birds, dehydration in acute cases, reduction in egg production and difficult breathing (Swayne *et al.* 2020). At the necropsy examination, a few gross lesions have been reported. It includes omphalitis, peritonitis, unabsorbed yolk sacs, severe enteritis, small white necrotic foci in the liver leading to swollen liver and sometimes bronze discoloration. Splenomegaly, irregular and misshapen ova, arthritis, pericarditis and yellow turbid fluid in peritoneal cavity are the significant findings during necropsy examination (Gedeno *et al.* 2022; Shakir *et al.* 2024).

Salmonella is a foodborne bacterium which causes public health problems around the globe, especially in developing countries, leading to huge economic losses for poultry farmers. Poultry and poultry products are mostly contaminated with this bacterium, because *Salmonella* is present in gastrointestinal tract causing contamination of carcass during slaughtering and leading to its transmission to the consumers. Common serovars till date are *Salmonella enteritidis* and *S. typhimurium* which are responsible for zoonosis (Borges *et al.* 2019; Sedeik *et al.* 2019). Non-host specific *Salmonella* serotypes may be transmitted through the intake of contaminated poultry products, which poses a serious health concern for the public. Salmonellosis, or *Salmonella* infection in humans, causes "food poisoning" leading to abdominal cramps, diarrhoea, vomiting and fever. However, these clinical symptoms and their severity are linked with the type of strain as well as the host's age and health status of both. The severity of the symptoms is higher in children, older adults, those with compromised immune systems, and pregnant females (Acevedo-Villanueva *et al.* 2021; Klochko 2021).

Traditional method for isolation of *Salmonella* such as culture is considered still as gold standard which requires enrichment or pre-enrichments steps. For this purpose, different media and biochemical tests are necessary for confirmation. This whole process takes about 5-7 days which is not feasible for rapid detection of such zoonotic diseases. Some molecular methods like polymerase chain reaction (PCR), Multiplex PCR, Real-time and nested PCR are recently used for detection of *Salmonella*. Besides these, isothermal nucleic acid amplification techniques are widely used like Nucleic acid Sequence-Based Amplification (NASBA) and Loop-mediated isothermal amplification (LAMP) assay (Naushad *et al.* 2023).

This study was planned to investigate the epidemiology and patho-morphology of the avian salmonellosis in backyard and commercial poultry. Furthermore, this study will also help to layout the public health safety protocols by detecting *Salmonella* contamination in poultry meat through rapid and sensitive methods. In Pakistan, to the best of our knowledge, there is no published data regarding isolation of *Salmonella* having zoonotic potential in backyard and commercial poultry by Loop-mediated isothermal amplification (LAMP) assay and about sensitivity and specificity of different techniques in comparison to each other. No previous study has been conducted regarding Salmonellosis in backyard poultry in the neglected pockets of the Faisalabad. There is a lack of quantitative information regarding the load of various *Salmonella* serovars in backyard poultry in the region.

Materials and Methods

Ethical statement

This study was conducted by following all the guidelines of National Biosafety Rules (2005); Punjab Biosafety Rules (2014); Punjab Animal Health act (2019) and Bioethics protocols by the Institutional Biosafety committee, University of Agriculture, Faisalabad, Pakistan (vide letter D. No. 3674/ORIC, Dated: 30-08-2021).

Study area and sampling method

For sample collection, simple random sampling technique a described by Thrushfield (2007) was applied to collect the samples from district Faisalabad including its five tehsils including Faisalabad, Chak Jhumra, Jaranwala, Samundri, and Tandliyanwala. For the expected prevalence of *Salmonella*e, a previous study published by Bhatti *et al.* (2013) was taken as reference (43%) for Pakistan. The following formula described by Thrushfield (2007) was used for estimation of sample size whereas n = required sample size, z = 1.96 and L/d = 0.05 (the desired level of precision/accuracy).

$$n = 1.96^2 \times Pexp(1 - Pexp)/d^2$$

The calculated sample size was n = 376, those were collected for the said purpose.

Sample collection

A total of 376 samples were collected from both

commercial and backyard poultry including broiler (n = 209), layer (n = 60) and non-descript (107) birds from the study area *i.e*., district Faisalabad. A well-structured questionnaire was designed to collect information from the farmers about the flocks. Total three (03) birds were sampled from each farm for DNA extraction and histopathology, separately. Liver samples were collected and half were stored in normal saline for culture examination. For the molecular tests, the other half of the liver was stored in zip bag at -20°C till further analysis.

Culture isolation

For culture isolation, liver samples were enriched in Rappaport-Vassiliadis Broth (Oxide, Basingstoke, UK) and then cultured on different medias including Blood agar, *Salmonella* Shigella (SS) agar (Oxoid, Basingstoke, UK) and Xylose Lysine Deoxycholate (XLD) agar (Oxoid, Basingstoke, UK) for selective growth of *Salmonella*. These plates were incubated at 37°C for 24 h to obtain specific colonies of the target organism for morphological identification. Gram staining was performed as described previously by the Tripathi and Sapra (2021). The stained smears were observed using 100X oil immersion lens under light microscope.

Molecular identification through PCR and LAMP

DNA extraction

Collected samples (tissues/culture) were further processed for DNA extraction. DNA extraction was carried out through commercial DNA extraction kit ZOKEYO (HDP408 Wuhan Zokeyo Biotechnology Co., Ltd., China). The supernatants were collected and stored at -20ºC for polymerase chain reaction.

Polymerase chain reaction (conventional and multiplex)

PCR reactions were carried out from extracted DNA and amplification protocol described by Alzwghaibi *et al.* (2018) was applied. The Primers used for conventional and multiplex PCR have been detailed in Table 1 and 2 for the genus *Salmonella* and serovars included in this study. Briefly, all the PCR reactions were performed in a total volume reaction of 25 μL comprised of 12.5 μL Master Mix (Zokeyo, UK), 1 μL of each forward and reverse primers, 2 μL of DNA template, deionized nuclease free distilled water. For positive control, *Salmonella* positive sample was used, while nuclease-free water was used as negative control. The PCR products were electrophoresed in 1% agarose for 40 min at 100 volts. The gels were stained with ethidium bromide and placed in UV tray for visualization of DNA band on Gel Doc through image lab software (Bio-Rad Gel doc EZ imaging System).

Loop-mediated isothermal amplification assay (LAMP)

The *Salmonella* invasion gene (*inv*A) was used as target for designing LAMP primers. Six LAMP primers *i.e*., F3, B3, BIP, FIP, LF and LB were used in the current study for the LAMP assay as shown in Table 1 described by Youn *et al.* (2017). Briefly, The LAMP reaction mixed in a total volume of 25 μL was based on the commercial Loop amp® DNA Amplification Kit (Eiken Chemical Co., Ltd., Japan), consisted of 1× thermal buffer, 6 m*M* of $MgSO_4$, 0.8 *M* of betaine, 1.6 m*M* of deoxyribonucleotide triphosphate (dNTP), 0.2 μM of each outer primer, 1.6 μM of each inner primer, 0.8 μM of each loop primer, 8 U of Bst DNA polymerase (New England Biolabs®, Ipswich, USA) and 2 μL of DNA templates. The LAMP assay was carried out in water bath at 60°C for 1 h. In this protocol, 1% agarose gel was prepared and 3 μL of DNA product was run on agarose gel to observe results. A typical ladder like pattern was observed for positive samples.

Statistical analysis

The obtained data was subjected to Chi-square test using Minitab software package (Akers 2018) to estimate prevalence of avian salmonellosis and its correlation with associated risk factors. The association between both variables *i.e*., response and explanatory was estimated through binary logistic regression.

Results

Morphological identification of the isolated organism

Salmonella showed opaque, translucent, smooth, and round colonies on *Salmonella* Shigella (SS) agar, whereas on Xylose Lysine Deoxycholate (XLD) agar pink colonies with or without a black center were observed on XLD agar. On blood agar, greyish white colonies were observed as shown in Fig. 1. Gram's stain smears from suspected colonies showed Gram-negative rod-shape bacteria, or *Bacillus* as shown in Fig. 1.

PCR and LAMP results

All the selected primers were detected through different genes. Genus *Salmonella* was identified through the *inv*A gene at 284 bp (Fig. 2A). *S. typhimurium* was identified through the presence of the genes *rfb*J, *fli*C and *flj*B at 663 bp, 183 bp and 526 bp, respectively. *S. enteritidis* was detected through the genes *spv* and *sef*A at 250 bp and 310 bp, respectively. *S. pullorum* and *S. gallinarum* were detected through the presence of *hut*, *slg*C and *spe*C genes at 495 bp, 252 bp and 174 bp, respectively as shown in Fig. 2C. For the LAMP assay, six primers were used for *inv*A gene to detect genus *Salmonella* in this study (Fig. 2B) and was detected by gel electrophoresis. A ladder-like pattern

Table 1: Primers used for conventional PCR and LAMP assay for genus *Salmonella*

Primers	Target gene	Sequence	Product size (bp)	Reference
Primers for PCR for genus *Salmonella*				
ST139-F	*inv*A	GTGAAATTATCGCCACGTTCGG GCAA		Rahn *et al*. (1992)
ST141-R	*inv*A	TCATCGCACCGTCAAAGGAACC	284	
Primers for LAMP assay for genus *Salmonella*				
F3	*inv*A	TCCTGGTACTAATGGTGATGA		Youn *et al*. (2017)
B3		GCCTCGATCAAGATAAGACG		
FIP		AACACCAATATCGCCAGTACGATATGTTCGTCATTCCATTACCTAC		
BIP		TGACAGAATCCTCAGTTTTTCAACGATCGATAATGCCAGACGAAAG		
LF		GCGCGATCAGGAAATCAACC		
LB		CCTGCGGTATTGTTAATAACAACAC		

Note: Amplicon size (bp) was ladder like bands with variable sizes

Table 2: Primers used for Multiplex PCR for different *Salmonella* spp.

Primers	Target gene	Sequence	Product size (bp)	Reference
Primers used in Multiplex PCR for *S. typhimurium*				
Rfbj-F	*rfb*J	CCAGCACCAGTTCCAACTTGATAC	663	Lim *et al*. (2003)
Rfbj-R		GGCTTCCGGCTTTATTGGTAAGCA		
Flic-F	*fli*C	ATAGCCATCTTACCAGTTCCCCC	183	
Flic-R		GCTGCAACTGTTACAGGATATGCC		
Fljb-F	*flj*B	ACGAATGGTACGGCTTCTGTAACC	526	
Flbj-R		TACCGTCGATAGTAACGACTTCGG		
Primers used in Multiplex PCR for *S. enteritidis*				
S1	*spv*a	GCCGTACACGAGCTTATAGA	250	Soumet *et al.* (1999)
S4	*spv*	ACCTACAGGGGCACAATAAC		
SEFA2	*sef*Ab	TGTGACAGGGACATTTAGCG	310	Woodward and Kirwan (1996)
SEFA4	*sef*A	TGTGACAGGGACATTTAGCG		
Primers used in Multiplex PCR for *S. pullorum* and *S. gallinarum*				
Hut-F	*hut*	ATGTTGTCCTGCCCCTGGTAAGAGA	495	Lin *et al.* (2014)
Hut-R		ACTGGCGTTATCCCTTTCTCTGCTG		
SGP-F	*slg*C	CGGTGTACTGCCCGCTAT	252	Kang *et al.* (2011)
SGP-R		CTGGGCATTGACGCAAA		
Primers used in Multiplex PCR for *S. gallinarum*				
SG-F	*spe*C	GAT CTG CTG CCA GCT CAA	174	Kang *et al*. (2011)
SG-R		GCG CCC TTT TCA AAA CAT A		

was found on gel electrophoresis for positive samples, as shown in Fig. 2.

Overall prevalence of avian salmonellosis and its association to risk factors

The overall results in the study are showed that avian salmonellosis is prevalent (12.5%). In different tehsils, the prevalence of genus *Salmonella* was recorded in tehsil Faisalabad, Jaranwala, Samundri and Tandliyanwala as 12.81, 13.48, 14.81 and 7.14%, respectively. Statistically higher prevalence ($P < 0.05$) of *Salmonella* was recorded in Samundri (Fig. 3). Based on different housing system, overall prevalence of genus *Salmonella* was as; open (21.31%), semi-control (9.59%) and control (7.73%) and it was significantly different statistically ($P < 0.05$) (Fig. 3).

Samples were collected from different sources including poultry farms, diagnostic laboratory and retail market birds. Based on these sources, out of 290 samples collected from diagnostic laboratory, 16.21% were positive for avian salmonellosis while no sample was found positive from poultry farms and retail market birds (Fig. 3). Based on bird types, overall prevalence of avian salmonellosis (genus *Salmonella*) was recorded as; broiler (9.15%), layer (18.33%) and non-descript (19.63%). Highest prevalence was recorded in non-descript birds as shown in Fig. 3. Prevalence percentage of co-infection in avian salmonellosis was 11.58% and it was statistically significant (Fig. 3). Prevalence of avian salmonellosis (genus *Salmonella*) in different seasons was as; autumn (42.9%), summer (7.4%), winter (26.1%) and spring (17.0%). Highest prevalence was observed in autumn followed by winter, spring and summer season (Fig. 3). The data based on tehsils also indicated that no sample was found positive in tehsil Chak Jhumra. However, prevalence in other tehsils and its association with risk factors has been described below in detail.

Prevalence and its association to risk factors in Tehsil Faisalabad

The prevalence of co-infection along with salmonellosis in tehsil Faisalabad was 9.52%, while in those birds having no co-infection was 13.66%. The probability of avian salmonellosis in tehsil Faisalabad was 1.50 times higher as compared to other tehsils (Table 3). In the same area, based on different shed types, prevalence was recorded as; open

Table 3: Disease risk of avian salmonellosis in relation to risk factors in tehsil Faisalabad and Jaranwala

Risk Factors	Variables	Faisalabad						Jaranwala					
		Total	Positive	Negative	Prevalence (%)	Chi-Square	*P* Value	Total	Positive	Negative	Prevalence (%)	Chi-Square	*P* Value
Co-infection	Yes	42	4	38	9.52	0.51	*0.475*	24	5	19	20.83	1.52	*0.217*
	No	161	22	139	13.66			65	7	58	10.77		
Type of Housing	Control	95	7	88	7.37	6.72	*0.035*	53	1	52	1.89	15.82	*0.000*
	Open	66	14	52	21.21			27	9	18	33.33		
	Semi-control	42	5	37	11.90			42	2	7	22.22		
Flock size	50 to 3000	34	7	27	20.59	9.69	*0.021*	9	3	6	33.33	11.76	*0.008*
	3001 to 9000	46	9	37	19.57			20	6	14	30.00		
	9001 to 18000	35	6	29	17.14			9	1	8	11.11		
	Above 18000	88	4	84	4.55			51	2	49	3.92		
Season	Autumn	6	1	5	16.67	8.83	*0.032*	3	1	2	33.33	10.00	*0.019*
	Spring	60	9	51	15.00			24	5	19	20.83		
	Summer	108	8	100	7.41			61	5	56	8.20		
	Winter	29	8	21	27.59			1	1	0	100.00		

Note: $P < 0.05$ is considered significant

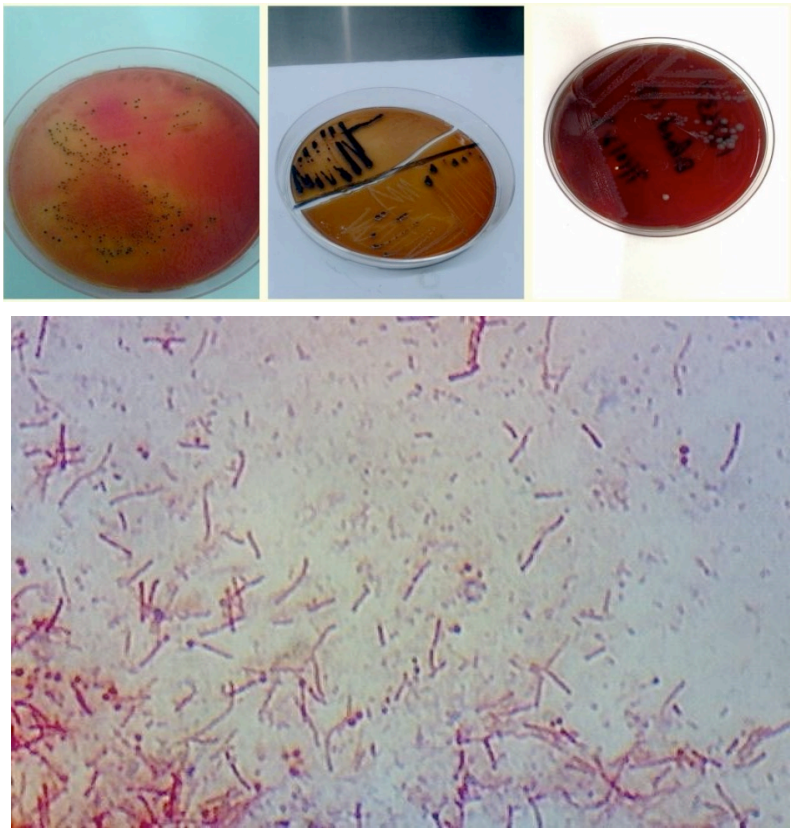

Fig. 1 (A): shows the colonies of *Salmonella* on different agars, **(B):** Gram staining of *Salmonella* showing gram negative rod at 100X

(21.21%), Semi control (11.90%) and control (7.37%). Highest prevalence was found in open sheds followed by semi-control and control houses, respectively. The probability of avian salmonellosis was 3.38 and 1.70 times higher in open houses and semi-control houses as compared to control houses (Table 3).

The percentage prevalence of avian salmonellosis in flock size ranging 50-3000, 3001-9000, 9001-18000 and above 18000 was 20.59, 19.57, 17.14 and 4.55%, respectively. The highest prevalence was recorded in flock size 50-3000 followed by 3001-9000, 9001-18000 and >18000, respectively. The probability of avian salmonellosis in different flock sizes *i.e.*, 3001-9000, 9001-18000 and >18000 was 0.94, 0.80 and 0.18, respectively (Table 3). The prevalence of avian salmonellosis in tehsil Faisalabad based on bird types has been recorded as broiler, layer and non-descript birds was 7.58, 28, and 20.90%, respectively (Table 3). The highest prevalence was recorded in layer (28%) followed by non-descript birds and broiler birds, respectively. Based on seasonal variation, data has shown

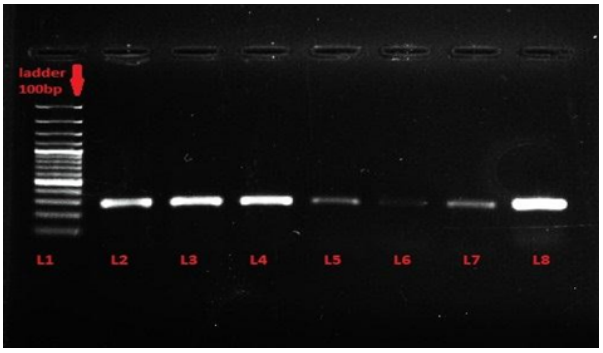

Fig. 2 (A): Agarose gel electrophoresis of PCR amplicons for genus *Salmonella* stained with ethidium bromide. Genus *Salmonella* positive samples are those which yielded an amplicon of the expected size of 284 bp). L1 = Molecular Ladder (100 bp), Lane 2 = control (+ve), Lane 3,4,5,6,7,8 = show positive samples

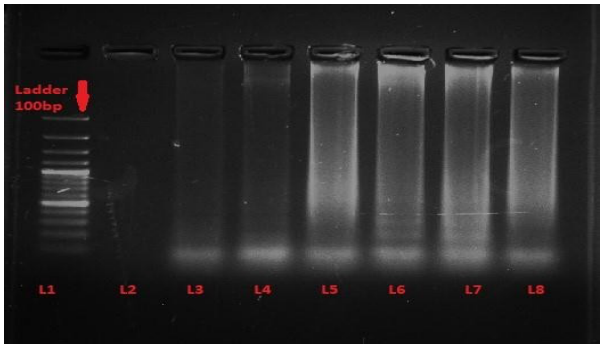

Fig. 2 (B): Ladder like Pattern of amplified products of genus *Salmonella* positive samples (*inv*A gene) upon LAMP reaction**.** Lane 1 = 100 bp DNA ladder. Lane 2 = negative control. Lane 3, 4, 5, 6, 7 = LAMP amplification showing positive sample *S.* Lane 8 = positive control

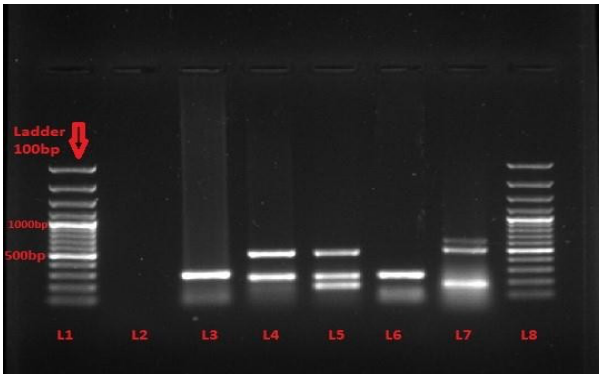

Fig. 2 (C): L1 = Molecular ladder (100 bp), Lane 2 = control (-ve), Lane 3 = shows positive sample for *inv*A gene (284 bp), Lane 4 = shows positive sample for *S. pullorum* genes *slg*C and *hut* (252 bp and 495 bp), Lane 5 = shows positive sample for *S. gallinarum* genes *spe*C, *slg*C and *hut* (174 bp, 252 bp and 495 bp), Lane 6 = shows positive sample for *S. enteritidis* gene *spv* (250 bp), Lane 5 = shows positive sample for *S. typhimurium* genes *fli*C, *flj*B and *rfb*J (183 bp, 526 bp and 663 bp), L8 = Molecular Ladder (100 bp)

that the prevalence of avian salmonellosis infection in winter, summer, spring and autumn was 27.59, 7.41, 15 and 16.67%, respectively. The highest prevalence was recorded in winter season (Table 3).

Prevalence and its association to risk factors in Tehsil Jaranwala

The prevalence of co-infection of avian salmonellosis was 20.83% while in those birds having no co-infection was 10.77% (Table 3) in this tehsil. The prevalence of avian salmonellosis infection in different sheds were as; open (33.33%), Semi control (22.22%) and control (1.89%). Highest prevalence was recorded in open sheds and probability of avian salmonellosis was 26 and 14.86 times higher in open and semi-control sheds as compared to control (Table 3).

The prevalence of avian salmonellosis in flock size ranging 50-3000, 3001-9000, 9001-18000 and above 18000 was 33.33, 30, 11.11 and 3.92%, respectively. The highest prevalence was recorded in flock size of 50-3000 followed by others (Table 3) and probability of avian salmonellosis in different flock size *i.e*., 3001-9000, 9001-18000 and >18000 was 0.86, 0.25 and 0.08, respectively (Table 3). Season bases prevalence of avian salmonellosis infection in tehsil Jaranwala during winter, summer, spring and autumn was 100% (one sample positive out of total one sample), 8.20, 20.83 and 33.33%, respectively. The highest prevalence was recorded in winter season (Table 3).

Prevalence and its association to risk factors in Tehsil Samundri

The prevalence of co-infection of avian salmonellosis was 7.14% while in those birds having no co-infection was 17.50% (Table 4). Based on different shed types were shown as: open (14.29%) and control (29.41%), while no sample was found positive in semi-control sheds (Table 4). Based on flock sizes ranging 50-3000, 3001-9000, 9001-18000 and above 18000, prevalence was 37.50, 10, 13.33 and 9.52%, respectively. The highest prevalence was recorded in flock size of 50-3000 (Table 4). Seasonal

Table 4: Disease risk of avian salmonellosis in relation to risk factors in tehsil Samundri and Tandliyanwala

Risk Factors	Variables	Total	Positive	Negative	Prevalence (%)	Chi-Square	*P* Value	Total	Positive	Negative	Prevalence (%)	Chi-Square	*P* Value
		Samundri						Tandliyanwala					
Co-infection	Yes	14	1	13	7.14	0.88	*0.348*	7	1	6	14.29	1.08	*0.299*
	No	40	7	33	17.50			7	0	7	0.00		
Type of Housing	Control	17	5	12	29.41	5.66	*0.059*	10	1	9	10.00	--	--
	Open	21	3	18	14.29			0	0	0	--		
	Semi-control	16	0	16	0.00			4	0	4	0.00		
Flock size	50 to 3000	8	3	5	37.50	3.94	*0.268*	0	0	0	--	--	--
	3001 to 9000	10	1	9	10.00			3	0	3	0.00		
	9001 to 18000	15	2	13	13.33			3	0	3	0.00		
	Above 18000	21	2	19	9.52			8	1	7	12.50		
Season	Autumn	9	4	5	44.44	8.21	*0.042*	1	0	1	0.00	--	--
	Spring	12	1	11	8.33			8	1	7	12.50		
	Summer	8	0	8	0.00			3	0	3	0.00		
	Winter	25	3	22	12.00			2	0	2	0.00		

Note: $P < 0.05$ is considered significant

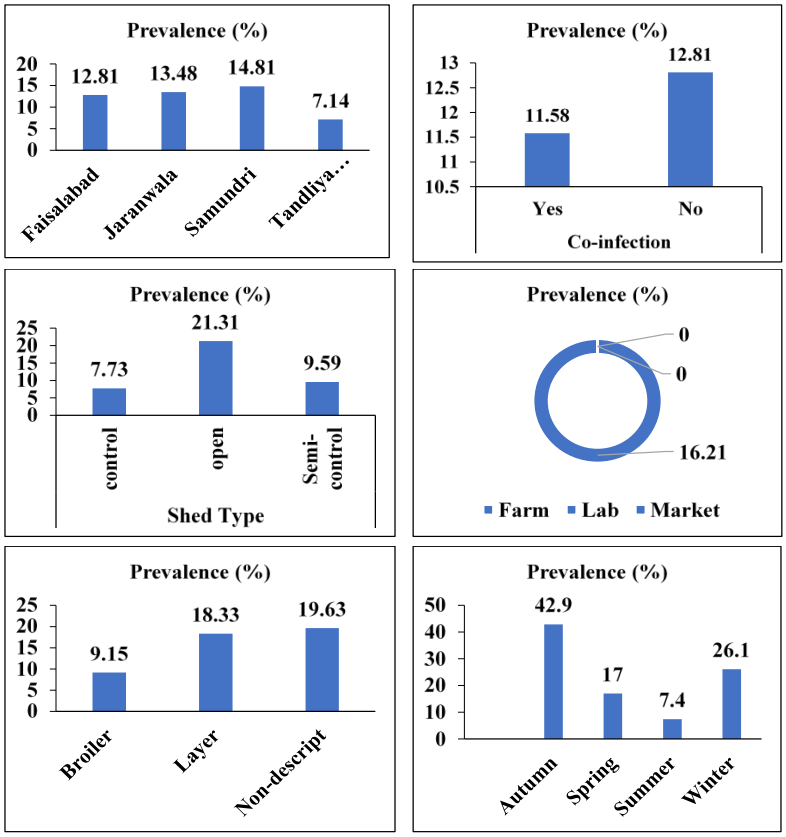

Fig. 3: Overall prevalence of avian salmonellosis

variation indicated that during winter, spring and autumn prevalence was 12, 8.33 and 44.44%, respectively. The highest prevalence was recorded in autumn season; however, no sample was found positive during summer season (Table 4).

Prevalence and its association to risk factors in Tehsil Tandliyanwala

The prevalence of co-infection of avian salmonellosis was 14.29%. The prevalence in different shed types was recorded in control (10%), while no sample was found positive in open and semi-control sheds. The prevalence based on flock size was recorded at 0% except above 18000, where it was 12.50%. Based on bird type, prevalence in broiler was 9.09%, while no positive birds were found in layer and non-descripts. Based on season in this tehsil, prevalence was 12.5% during spring, however, in all other seasons no positive case was recorded in birds (Table 4).

Table 5: Avian Salmonellosis infection in different type of birds in District Faisalabad

Tehsils of District Faisalabad	Type of birds	Total sample	Positive	Negative	Prevalence (%)	Chi-Square	Odds ratio	95% C. I		*P* value
								Lower	Upper	
Faisalabad	Broiler	66	5	61	7.58	26.12	3.55	3.02	4.28	*0.044**
	Layer	25	7	18	28.00		2.95	2.42	3.68	*0.021**
	Retail Market Birds	45	0	45	0.00		3.60	3.07	4.33	*0.041**
	Non-descript	67	14	53	20.90		2.90	2.37	3.63	*0.044**
Jaranwala	Broiler	57	5	52	8.77	26.73	4.31	3.78	5.04	*0.032**
	Layer	5	1	4	20.00		3.21	2.68	3.94	*0.022**
	Retail Market Birds	0	0	0	-		-	-	-	-
	Non-descript	27	6	21	22.22		2.75	2.22	3.48	*0.013**
Samundari	Broiler	26	4	22	15.38	23.02	2.68	2.15	3.41	*0.013**
	Layer	22	3	19	13.64		3.92	3.39	4.65	*0.031**
	Retail Market Birds	0	0	0	-		-	-	-	-
	Non-descript	6	1	5	16.67		3.20	2.67	3.93	*0.027**
Tandilyanwala	Broiler	11	1	10	9.09	8.75	3.12	2.59	3.85	*0.108*
	Layer	2	0	2	-		-	-	-	-
	Retail Market Birds	0	0	0	-		-	-	-	-
	Non-descript	1	0	1	-		-	-	-	-
Jhumra	Broiler	4	0	4	-	-	-	-	-	-
	Layer	6	0	6	-		-	-	-	-
	Retail Market Birds	0	0	0	-		-	-	-	-
	Non-descript	6	0	6	-		-	-	-	-

Note: $P < 0.05$ is considered significant

Table 6: Disease risk of Avian Salmonellosis infection with/without co-infection

Type of birds	Co-infection	Total	+ve	-ve	Prevalence (%)	Chi-Square	Odds ratio	95% C. I		*P* value
								Lower	Upper	
Broiler	Yes	50	8	42	16.00	16.23	2.51	1.98	3.24	*0.023**
	No	114	7	107	6.14		2.80	2.27	3.53	*0.104*
Layer	Yes	21	0	21	-	-	-	-	-	-
	No	39	11	28	28.21		1.86	1.33	2.59	*0.022**
Market Broiler	Yes	0	0	0	-	-	-	-	-	-
	No	45	0	45	-		-	-	-	-
Non-descript	Yes	24	3	21	12.50	10.91	2.66	2.13	3.39	*0.040**
	No	83	18	65	21.69		2.42	1.89	3.15	*0.026**

Note: $P < 0.05$ is considered significant

Prevalence in different poultry types and associated risk factors

Risk of avian salmonellosis infection in district Faisalabad: At the district level, the prevalence of avian salmonellosis has been recorded as follows: various tehsils of district Faisalabad includes Faisalabad, Jaranwala, Samundri, Tandlianwala and Chak Jhumra, where different poultry breeds have been calculated. In the district of Faisalabad, no sample was found positive for avian salmonellosis in retail market birds.

However, at tehsil level (Faisalabad), prevalence of salmonellosis in broiler, layer and non-descript was 7.58, 28 and 20.90%, respectively. The probability of broiler, layer and non-descript was 3.55, 2.95 and 2.90, respectively (Table 5). Highest prevalence of salmonellosis was recorded in layer birds. The pattern of prevalence based on bird types was different in tehsil Jaranwala, where highest prevalence was recorded in non-descript birds (22.22%), followed by layer (20%) and broiler (8.77%). The probability of non-descript, broiler and layer was 2.75, 4.31 and 3.21, respectively (Table 5).

In tehsil Samundri, prevalence of diseases in broiler, layer and non-descript was 15.38, 13.64 and 16.67%, respectively. Highest prevalence was recorded in non-descript while lowest prevalence was observed in layer birds (Table 5). In tehsil Tandilyanwala, the prevalence of disease in broiler was 9.09%. No samples were found positive for layer and non-descript birds, while in tehsil Chak Jhumra no sample was found positive for any type of the poultry bird (Table 5).

Associated prevalence of co-infection with avian salmonellosis: The prevalence of co-infection with avian salmonellosis in different birds including broiler and non-descript was 16 and 12.50%, respectively with probability of 2.51 and 2.66, respectively (Table 6), while no sample was found positive for co-infection in layer and retail market birds. The prevalence of salmonellosis in birds examined having no co-infection was 6.14% in broiler, 28.21% in layer, and 21.69% in non-descripts (Table 6). The highest prevalence of salmonellosis and its association with co-infection was recorded in broiler followed by non-descript.

Prevalence of avian salmonellosis associated with housing systems based on bird types: The prevalence of salmonellosis in broiler birds in different sheds were as;

Table 7: Disease risk of Avian Salmonellosis in different housing systems and Seasons

Type of birds	Parameter	Total sample	Positive	Negative	Prevalence (%)	Chi-Square	Odds ratio	95% C. I		*P* value
								Lower	Upper	
Shed Type (*P < 0.05* is considered significant)										
Broiler	Open	129	11	118	8.53	18.23	3.96	3.43	4.69	*0.012**
	Control	18	2	16	11.11		1.74	1.21	2.47	*0.027**
	Semi-control	17	2	15	11.76		2.23	1.70	2.96	*0.032**
Layer	Open	19	3	16	15.79	17.23	1.66	1.13	2.39	*0.029**
	Control	18	6	12	33.33		2.51	1.98	3.24	*0.040**
	Semi-control	23	2	21	8.70		2.92	2.39	3.65	*0.017**
Retail Market Birds	Open	28	0	28	-	-	-	-	-	-
	Control	17	0	17	-		-	-	-	-
	Semi-control	0	0	0	-		-	-	-	-
Non-descript	Open	5	0	5	-	21.86	-	-	-	-
	Control	86	18	68	20.93		2.22	1.69	2.95	*0.026**
	Semi-control	16	3	13	18.75		3.32	2.79	4.05	*0.009**
Season (*P < 0.05* is considered significant)										
Broiler	Autumn	6	2	4	33.33	28.34	3.12	2.59	3.85	*0.046**
	Spring	63	6	57	9.52		2.52	1.99	3.25	*0.023**
	Winter	30	6	24	20.00		3.17	2.64	3.90	*0.043**
	Summer	65	1	64	1.54		2.47	1.94	3.20	*0.046**
Layer	Autumn	8	3	5	37.50	16.14	3.88	3.35	4.61	*0.034**
	Spring	25	6	19	24.00		2.78	2.25	3.51	*0.024**
	Winter	11	2	9	18.18		3.69	3.16	4.42	*0.032**
	Summer	16	0	16	-		-	-	-	-
Retail Market Birds	Autumn	0	0	0	-	-	-	-	-	-
	Spring	0	0	0	-		-	-	-	-
	Winter	0	0	0	-		-	-	-	-
	Summer	45	0	45	-		-	-	-	-
Non-descript	Autumn	6	1	5	16.67	19.64	2.69	2.16	3.42	*0.110*
	Spring	22	5	17	22.73		4.14	3.61	4.87	*0.030**
	Winter	17	3	15	17.65		4.87	4.34	5.60	*0.028**
	Summer	62	12	50	19.35		2.52	1.99	3.25	*0.039**

open (8.53%), Semi control (11.76%) and control (11.11%). Highest prevalence was recorded in semi-control shed followed by control and open sheds (Table 7). The prevalence of salmonellosis in layer birds in different sheds were as; open (15.79%), Semi control (8.70%) and control (33.33%). Highest prevalence of salmonellosis was recorded in layer birds maintained in control houses followed by open hoses and semi-control, respectively (Table 7).

The prevalence of salmonellosis in non-descript birds in different poultry houses were as; semi control (18.75%) and control (20.93%). No sample was found positive for salmonellosis in open shed in non-descript birds. Highest prevalence was documented in control houses followed by semi-control and open houses. The probability of non-descript birds in control and semi-control sheds was 2.22 and 3.32, respectively (Table 7).

Seasonal prevalence of avian salmonellosis based on types of poultry: No sample was found positive for salmonellosis in retail market birds in different seasons. The probability of broiler birds in different seasons *i.e.*, autumn, spring, winter and summer was 3.12, 2.53, 3.17 and 2.47, respectively (Table 7). The prevalence of avian salmonellosis infection in broiler birds in different seasons was recorded as follows; winter (20%), summer (1.54%), autumn (33.33%) and spring (9.52%). The highest prevalence was documented in autumn season followed by winter, spring and summer, respectively. Statistical analysis of data indicated that the prevalence of avian salmonellosis is statistically significant at different seasons among broilers.

The prevalence of avian salmonellosis in layer birds in different seasons were as follows; winter (18.18%), autumn (37.50%) and spring (24%) while no prevalence was observed in summer season. Highest prevalence was recorded in autumn season followed by spring season and winter season, respectively (Table 7) and it was statistically significant. The highest prevalence was recorded in spring season followed by summer, winter and autumn season, respectively.

The prevalence of salmonellosis infection in non-descript birds in different seasons was as follows; winter (17.65%), summer (19.35%), autumn (16.67%) and spring (22.73%) and it was statistically significant. The probability of non-descript birds in seasons like summer, winter, spring and autumn was 2.52, 4.87, 4.14 and 2.69, respectively (Table 7).

Discussion

Backyard poultry farming is a source of significant income for rural farmers and smallholder in Pakistan. Like commercial poultry production, backyard poultry farming is essential to both economic growth and food security (Sharma 2010). Backyard chicken farming accounts for a smaller percentage of Pakistani agriculture. As per Pakistan

Economic Survey 2023–2024, 95.50 million units than commercial chicken farming *i.e.,* 1968.71 million units. However, the Government of Pakistan is committed to enhance this farming and will provide five million birds to concerned citizens at subsidized rates through the "Prime Minister's Initiative for Backyard Poultry Project," which was started in 2019 (GOP 2022, 2023).

The objectives of the current research are to study epidemiology of avian salmonellosis in commercial and backyard poultry and its association to various risk factors along with zoonotic importance. Conventional and molecular techniques were compared to find out the most suitable approach for the rapid identification of prevalent species of *Salmonella* in poultry. This study revealed that typical black centered colonies of *Salmonella* with reddish zone on XLD were observed due to change in color of media, while black centered colonies were observed on SS agar. The investigation showed that *Salmonella* on XLD typically produced pink colonies with or without black centers and the same findings have been previously endorsed by Sedeik *et al.* (2019). Later all these positive samples were confirmed through PCR for confirmation.

A total of 376 samples from district Faisalabad were collected and out of these 47 samples (12.5 %) samples were found positive for genus *Salmonella* through PCR, (*inv*A gene). Our investigation focused on identifying the *Salmonella* genus that codes for a type III secretion system, using *inv*A gene. It is often utilized for molecular based identification of *Salmonella* genus in short time because of its conserved nature, as opposed to prolonged and time-consuming enrichment techniques (Abdel-Aziz 2016; Ahmad *et al.* 2020). It is widely accepted that the international standard method for detecting the *Salmonella* genus is amplification of the *inv*A (El-Feky *et al.* 2014). Some previously reported results also endorse the significance of this gene as reported by Ahmad *et al.* (2020), who reported that the *inv*A gene was amplified in 94.4% isolates, and similar findings were reported by Karatuğ *et al.* (2018), who stated that 94.7% samples were positive for *Salmonella* strains via this gene.

In terms of prevalence, current study findings indicated higher prevalence when compared with previously reported by Shanmugasamy *et al.* (2011) having the prevalence of 8.3% and it was lower when compared to Kaushik *et al.* (2014), who reported 23.7% prevalence. In another study, Jibril *et al.* (2020) reported the sample level prevalence *i.e.*, 15.9% of *Salmonella* in Nigeria which is closer to current study. Ansari-Lari *et al.* (2014) reported that the prevalence of *Salmonella* spp. in the broiler flocks in Shiraz; southern Iran was 22.50% through culture. Many other studies have reported relatively higher prevalence including Andoh *et al.* (2016), Odoch *et al.* (2017) and Eguale (2018) who reported higher prevalence in poultry farms in sub-Saharan countries such as Ghana (44.0%), Uganda (20.7%) and Ethiopia (14.6%), respectively (Andoh *et al.* 2016; Odoch *et al.* 2017; Eguale 2018). Likewise, in developing Asian countries, Barua *et al.* (2012) and Lettini *et al.* (2016) reported prevalence of 46.3 and 18% in central Vietnam and Bangladesh, respectively. In another study in Pakistan, Ahmad *et al.* (2020) reported overall prevalence of *Salmonella* as 21.6% in poultry through PCR in Peshawar. Formerly, a comparable study was done in Pakistan, in the area of Kohat and authors found 26.6% prevalence of *Salmonella* in poultry birds (Asif *et al.* 2017).

Our research verified the presence of all genes, *i.e*., *fliC*, *rfbj* and *fljB* in birds positive for *S. typhimurium*. Similar findings have been reported by Lim *et al.* (2003), who also used three primer sets of genes showed concurrent presence of all three genes which were specific and essential to identify *S. typhimurium* serovar and same has been endorsed in another study conducted by Amini and Amini (2019). These outcomes are in accordance to aforementioned research conducted by Moosavy *et al.* (2015) to detect and identify *S. typhimurium* by molecular test. For specific detection of *S. Enteritidis*, *spv* gene (plasmid virulence gene) and fimbrial antigen gene *i.e*., *sef*A were used in the current study and it revealed the presence of *spv* gene in all the samples positive for *Salmonella Enteritidis* but *sef*A gene was not detected. The results of previously reported studies are consistent with our findings (Borges *et al.* 2013; Dousandeh *et al.* 2016).

Multiplex PCR results in the present study showed amplified segments of the hut, *slg*C and *spe*C genes, which allows for a very strong differentiation between *S. gallinarum* and *S. pullorum*. All the genes were positive in the present study. A previous study was in line with our results conducted by Alzwghaibi *et al.* (2019) in which the PCR results for *S. gallinarum* and *S. pullorum* in chickens showed a distinct band at 252 bp for the *Slg*C gene, which is present in *S. gallinarum* and *S. pullorum.* Alzwghaibi *et al.* (2019) performed PCR in chickens and results were also similar to our study in which using a specific set of primers for the *spe*C gene, it was discovered that the band at 174 bp is unique to *S. gallinarum* and not present in *S. pullorum* or other serovars. This showed a differentiation between *S. pullorum* and *S. gallinarum.* These results of multiplex PCR are also in agreement with the previous research reported by Kang *et al.* (2011) and Li *et al.* (2014).

At the district level, highest prevalence was found in tehsil Samundri. In tehsil Faisalabad the highest prevalence was present in layer birds (28%). In non-descript birds, highest prevalence (22.22% and 16.67%) was in tehsil Jaranwala and Samundri. Highest prevalence of broiler (15.38%) was in tehsil Samundri which is lower than the reported in the region (22.50%) (Ansari-Lari *et al.* 2014). Similar findings have been reported by Tagar and Qambrani (2023) in Hyderabad and Jamshoro district of Pakistan and reported 97.3% of prevalence of *Salmonella* in meat samples. A comparable study carried out in Nepal, by Mahato (2019), where it was discovered that 41.3% of the meat samples were contaminated with *Salmonella*. Another study conducted in the Mymensingh and Jamalpur districts

of Bangladesh where 30% samples were found positive through PCR for *Salmonella* (Talukder *et al.* 2021). In central Ethiopia, in the towns of Bishoftu and Adama, prevalence of *Salmonella* was recorded as 16.14% (Kebede and Duga 2022). Sedeik *et al*. (2019) investigated the prevalence of *Salmonella* in El-Gharbia, El-Behera, Kafr-Elshikh, Alexandria, Marsamatroh Provinces in Egypt and recorded 7.5% prevalence which is lower than this study.

On the basis of housing, in broilers, the highest prevalence was found in semi-control houses (11.76%), in layer birds, highest prevalence was in control houses *i.e*., 33.33%, in non-descript birds, prevalence was highest in control system which was 20.93%. Findings of Davies and Breslin (2003), Pereira *et al.* (2010) and Shittu *et al.* (2014) revealed that infectious pathogens move from flock to flock in birds raised in poorly maintained housing. In Pakistan, the widespread use of antibiotics in commercial production system of poultry may possibly be a significant factor in the development of bacterial microorganism resistance (Ansari *et al.* 2014). It is clear that backyard chickens do not often expose to antibiotics, but they could be connected to human beings and other species by a variety of channels and can freely pick feed from unhygienic areas and thus can be exposed to resistant bacterial agents through feces (Shoaib *et al.* 2016). A prior Japanese study revealed that flocks raised in windowless poultry sheds had a considerably greater frequency of *Salmonella* than those flocks raised in windowed poultry houses (Sasaki *et al.* 2012; Samper-Cativiela *et al.* 2023).

In this current study on the basis of birds, the prevalence of *Salmonella* was 9.15% in broiler, 18.33% in layer and 19.63% in non-descript birds. No sample was found positive in retail market birds. It was recorded that the highest prevalence was in non-descript birds. In non-descript birds, the highest prevalence was in spring (22.73%). These findings are very helpful in highlighting the threats of avian salmonellosis in all sectors of poultry industry around the globe and specifically in Pakistan. Akhtaruzzaman *et al.* (2020) conducted a study in Bangladesh on layers and it was found that the overall prevalence of *Salmonella* was 15.6%. Comparably, 21.09% of the *Salmonella* isolates were found in backyard hens, while 35.06% were reported in commercial broilers (Shoaib *et al.* 2016). It can be described that mode of nutrition might be the cause of diversity and variations in prevalence of enterobacteria in backyard and commercial broilers (Saliu *et al.* 2012). This finding could be attributed to variations in geographic regions, composition of feed, as well as management circumstances, especially those given during the embryonic stage or the primary days of lifespan (Nghonjuyi *et al.* 2015). The overall seroprevalence of salmonellosis in broiler and layer was 42.67% described by Rani *et al.* (2022). Another molecular study conducted by Tiwari *et al.* (2022) showed that the overall prevalence of *Salmonella* was 13.33% and 36.36% in broiler and layer poultry farms, respectively. In this study, *inv*A gene was targeted through PCR which has amplified product size of 284 bp as in our study for genus *Salmonella*.

Seasonal influence also has been recorded on the overall prevalence of *Salmonella* in district Faisalabad. In broiler, the highest prevalence was recorded in autumn season (33.33%) and lowest was in summer which is 1.54%. In layer birds, the highest prevalence was in autumn *i.e*., 37.50%. Rani *et al.* (2022) described that avian salmonellosis in commercial broilers in Bangladesh was significantly higher in summer season *i.e*., 66.15% as compared to winter (27.50%) and rainy (31.11%) season, respectively. Roshdy *et al.* (2020) reported the highest prevalence of *Salmonella* in summer season. He further reported that during winter, isolation rates for chicks 13.3-20%, while it was 6.6% in the autumn. Temperature could play a significant role in *Salmonella*'s survival and growth. Warm temperatures create a favorable condition for *Salmonella* to develop and multiply (Soltan *et al.* 2009; Naurin *et al.* 2013).

Flock size also contributes as a risk factor for avian salmonellosis, while a greater flock size may be associated with an increased risk of being infected with *Salmonella* (Namata *et al.* 2008). Variable results were recorded in this current study *i.e*., farms with the smallest flock size (50-3000) had the highest prevalence as compared to larger flock size. It is difficult to explain these differences, though; carrier birds might be a possibility. According to several research studies (Huneau-salaün *et al.* 2009; Im *et al.* 2015), the number of chickens in a flock is a significant risk factor for *Salmonella* infection. Our findings align with this assumption. Differences in tehsils were also related to contamination of *Salmonella*. Other complex factors could be an issue here, but the tehsils and flock size association seem to be the most contributing risk factor.

Conclusion

From the current study it has been concluded that the *Salmonella* is prevalent in backyard poultry flocks which is a possible source of avian salmonellosis in commercial flocks as well as posing serious health risks to the public in terms of zoonotic strains. The variations in occurrence of *Salmonella* observed in the current study may be explained by variations in the research's location, duration, and season, sample size, work quality, types of samples, immune status of poultry birds, factors related to method geographical distribution, management systems of nations and poultry farms *i.e*., sanitary, biosecurity and hygiene, housing systems and resources available at laboratories.

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Author Contributions

NW and STG conceived idea, executed and recorded the results throughout the study period. NW, MKS, ZUDS and STG all were actively involved for the smooth running of this research, write up and data analysis *etc*.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

All the data presented in this manuscript and related to this research is available with the authors and can be provided on demand.

Ethical Approval

This study was conducted by following all the guidelines of National Biosafety Rules, 2005; Punjab Biosafety Rules 2014; Punjab Animal Health act 2019 and Bioethics protocols by the Institutional Biosafety committee, University of Agriculture, Faisalabad, Pakistan (vide letter D.No.3674/ORIC, Dated: 30-08-2021).

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