Evidence of Salmonella enterica *in Pakistani backyard poultry breeds: isolation, molecular characterization and pathology*

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Salmonella enterica,
Zoonoses.

Summary
The present study evaluated the presence of *Salmonella enterica* in Pakistani backyard poultry. A total 48 chickens from 4 backyard poultry breeds with the clinical presentation of *S. enterica* infection were randomly selected from villages in the Punjab Province. Cloacal swabs from live poultry and liver samples from the dead birds were collected for bacterial culture and biochemical identification. Liver and spleen samples from dead birds were evaluated for gross and histopathological changes. Bacterial isolates were subjected to PCR and sequencing of ratA gene. Biochemical identification revealed 5/48 (10.42%) chickens positive for *S. enterica*. Gross pathology included enlarged, discoloured and congested liver and congested spleen. Histopathology demonstrated congestion of sinusoidal capillaries, cellular swelling and cellular / ballooning degeneration, congestion of central hepatic vein, granular hepatocytic cytoplasm and the presence of variable-sized vacuoles in hepatocytes. The PCR yielded a *S. enterica* specific amplicon (1047 bp). All liver samples that were positive for *S. enterica* by biochemical tests, were also positive by PCR. The ratA gene sequencing revealed a close resemblance with *S. enteritidis* isolates from humans. The present study highlights zoonotic risk from backyard poultry and suggests that PCR can be used as an alternate method for rapid detection of Salmonella serovars.

Introduction

Backyard rearing of birds provides the most cost-effective source of eggs and meat, offering cash income to rural people (Mandal *et al*. 2006; Sharma 2010). Approximately 91 million birds constitute the backyard poultry sector of Pakistan (Anonymous, 2021). The unique nature of backyard and indigenous poultry, its key role in the rural economy in provision of protein to rural masses and its little to no biosecurity demand exploratory and investigative studies to ascertain the presence of zoonotic pathogens in the backyard poultry (Lyer 1950; Dessie and Ogle 2001; Sarkar and Golam 2009; Dessie *et al*. 2011).

Common backyard poultry breeds in Pakistan are Aseel, Black Australorp, Desi, Fayoumi, Naked Neck, Rhode Island Red (RIR), and numerous crossbreeds (Performance Report, non-dated). Fayoumi and RIR are the two most commonly reared backyard poultry breeds in Pakistan. A recent study conducted in Pakistan has indicated that Fayoumi and RIR are kept mainly for eggs, mothering behavior, broodiness and income generation (Sadef, 2015). Moreover, these breeds are liked due to their scavenging behavior and utilization of kitchen left overs. A previous study comparing Fayoumi and RIR have indicated that Fayoumi chickens reach sexual maturity earlier compared to RIR (Amer, 1965). Published literature suggests that RIR demonstrates greater egg

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production, better day-old-chick weight and better feed conversion ratio compared to Fayoumi (Amer 1972; Khawaja *et al*. 2012).
A crossbred of Fayoumi and RIR has lower mortality, greater egg production and better weight gain (Khawaja *et al*. 2012).

A recent study has suggested that the Backyard Poultry Initiative introduced recently by the Pakistani government to supply five million chickens to people for economic empowerment is likely to lead to the emergence of zoonotic infections, as multiple inexperienced people are adopting this scheme (Anonymous, 2021; Ahmed *et al*. 2021).

Salmonellosis is a zoonotic disease that causes gastroenteritis, diarrhea, and systemic typhoid fever in humans (McGhie *et al*., 2009).

Chicken is a natural host for *Salmonella enterica* but the bacterium is also reported in turkeys, quails, pheasants, sparrows and parrots (Shivaprasad and Barrow, 2008).
Salmonella enterica is evolving to expand the host range and hence constitute a risk for zoonotic infections (Liu *et al*., 2002).
A recent global review has suggested that annually ~ 93 million cases of gastroenteritis and 0.155 million deaths are linked to Salmonella (Castro-Vargas *et al*., 2020).
In the United States alone till August 2021, outbreaks of human salmonellosis linked to backyard poultry have resulted in 863 known illnesses, 209 known hospitalizations, and 2 known deaths (CDC, 2021). Moreover, 25% of the affected people were children < 5 years, highlighting the need to protect the young generation from the pathogen (CDC, 2021). Salmonella has also been reported in both poultry and human cases in European countries including Italy (Guerrini *et al*. 2021; Di Marcantonio et al. 2022). In Pakistan, exploratory studies to ascertain the presence of Salmonella in backyard poultry and establish its link to the outbreaks in humans are needed.

Salmonellosis is diagnosed with the help of clinical signs, necropsy, histopathology, and serology (Porter Jr 1998).
Clinical signs of Salmonellosis in chickens are well documented (Shivaprasad, 2000). Gross pathology of *Salmonella enterica* in poultry include discoloration of liver, spleen, and kidneys along with mottling, focal necrosis, hemorrhages and nodular abscess (Habib-ur-Rehman *et al.* 2004).
Histopathological indications of *Salmonella enterica* include diffused hepatitis, focal necrosis, fatty changes and focal infiltration of lymphocytes in liver, splenitis, oophoritis, salpingitis and necrosis of myocardium along with fibrosis (Chauhan, 1996; Shivaprasad, 2000).

Salmonella enterica infection in poultry leads to early chick mortality, decreased fertility, and low egg production (Shivaprasad, 2000). Morbidity and mortality in Salmonella-affected chickens depends on their age, health status, secondary infections, as well as the management conditions of flock. Mortality in chickens can range from 10-100% (Hall *et al*. 1949; Wong *et al*. 1996).

The modes of transmission for *Salmonella enterica* include infected carrier birds, poor sanitation, egg-eating, wound-pecking or cannibalism on infected birds (Hinshaw *et al*. 1926; Williams *et al*. 1968). The vertical / trans-ovarian transmission of *Salmonella enterica* in chickens is well-known (Beaudette 1925; Beach and Davis 1927) and is a serious threat to the backyard poultry production as this leads to huge economic losses to poultry farmers.

In a bacterio-pathological investigation among dead birds in Bangladesh, Salmonella was found to be the most common disease affecting following classes of chickens: adult layers (53.25%), brooding hens (14.55%), growing hens (16.10%), and pullets (16.10%) (Rahman *et al*. 2004).
A comparative study of *Salmonella enterica* infection in commercial and local chicken has found that the local chickens are equally susceptible to the infection as much as are the commercial chickens (Mdegela *et al*. 2002).

In Pakistan, *Salmonella enterica* serovars including *Salmonella enterica* subspecies enterica serovar Enteritidis (hereafter referred to as *Salmonella* Enteritidis) have been reported from commercial poultry but scant data about the presence of this bacteria in the backyard poultry breeds of Pakistan are available (Shakir *et al*. 2021; Siddique *et al*. 2021). The present study was designed to determine the presence of the *Salmonella enterica* in four backyard poultry breeds (Fayoumi, RIR, Naked Neck, and Reciprocal crossbred of Fayoumi and RIR) in the Jhelum District of the Punjab Province of Pakistan. Another objective was to evaluate PCR as a rapid diagnostic tool for the detection of *Salmonella enterica* from the local backyard poultry breeds.

Materials and methods

Study area

Jhelum is enriched with backyard and small-scale village poultry holdings (Figure 1).
Laboratory procedures were performed at the Department of Pathology and the Institute of Microbiology, Faculty of Veterinary Science, University of Veterinary and Animal Sciences, Lahore, Pakistan.

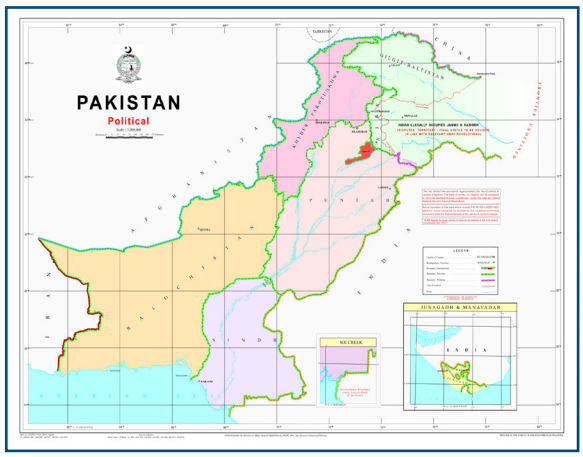

Figure 1. *The Jhelum District (red) in the Punjab Province of Pakistan is rich in backyard poultry holdings.*

Experimental Birds

A total of 48 chickens, 12 from each breed: Fayoumi, RIR, Naked Neck, and Reciprocal crossbred of Fayoumi and RIR were selected from different farms and the village holdings of Jhelum based on clinical signs consistent with Salmonellosis.

Sample Collection and Sample Processing

Cloacal swabs were collected in a commercially available Rappaport-Vassiliadis Broth (Oxide, Basingstoke, UK) for the enrichment and were further cultured on Brilliant Green Agar, for the selective growth of salmonella, and incubated at 37ºC for 24 hours to obtain specific colonies of the target organism for morphological and biochemical testing. Liver samples were collected in plastic zip bags and briefly stored at 4ºC until DNA extraction was done. At necropsy liver and spleen samples were collected in 10% neutral buffered formalin and transported to the Histopathology Laboratory of the Department of Pathology for microscopic evaluation.

Pathological study

Gross and histopathological studies were performed as per standard protocols. The prepared slides were observed under a light microscope at 10X and 40X (Culling *et al*. 2014).

Bacterial Culture

The colour and shape of bacterial colonies on Brilliant Green Agar were observed to ensure that they were consistent with *Salmonella enterica*.

Morphological characterization

Gram staining was done as described earlier (Coico, 2006).

The stained smears were observed under a microscope (Model: CX33RTFS2, Olympus®, Center Valley, PA, USA) with a total magnification of 1000X.

Biochemical characterization

Standard biochemical tests for the identification of *Salmonella enterica* such as triple sugar iron, indole fermentation, Voges Proskauer, methyl red and dulcitol fermentation tests were performed as described previously with minor modifications (Merck Microbiology Manual, 2005; Andrews *et al*. 2022).

Molecular characterization

The samples positive for Salmonella by biochemical testing were further evaluated by a PCR targeting ratA gene of *Salmonella enterica* (Batista *et al*. 2013). The PCR amplicons were sequenced and the sequence was deposited in the GenBank.

DNA extraction

DNA was extracted by a commercial kit (Favorgen, Ping-Tung, Taiwan) following the manufacturer's instructions.

Polymerase chain reaction

PCR assay targeting the ratA gene of *Salmonella enterica* was performed by using a reaction mixture containing 2µl each of Forward (5'-GACGTCGCTGCCGTCGTACC-3') and Reverse (5'-TACAGCGAACATGCGGGCGG-3') primers (10µM), 1µl of DNA template (at least 10 ng/ µl), 12 µl of Thermo Scientific Dream Taq (Life Technologies Inc., Carlsbad, CA, USA) master mixture and nuclease-free water up to 25 µl.

Cycling conditions were initial denaturation at 94°C for 3 minutes, followed by 25 cycles of denaturation at 94°C for 1 minute, Annealing at 63°C for 30 seconds, and extension at 72°C for 60 seconds, with a final extension at 72°C for 5 minutes (Batista *et al*. 2013).

While 1µl of DNA isolated from a Salmonella positive sample was used as an internal positive control, the same volume of nuclease-free water was used as a no template control.

Results were analyzed by running PCR product along with a 1 kb ladder (GeneRuler, ThermoFisher Scientific, Waltham, MA, USA) through gel electrophoresis at 4 V/cm for 60 minutes in a 1% (w/v) agarose gel stained with ethidium bromide followed by imaging in a gel documentation system (Major Science UV Transilluminator, Saratoga, CA, USA).

Gene sequencing

The PCR product was purified using a commercial kit (FavorPrep™ Gel Purification Mini Kit, Ping-Tung Agricultural Biotechnology, Ping-Tung, Taiwan) following the manufacturer's instructions. The sequence was obtained via the services of a commercial vendor. The sequence was analyzed using a publically available online BLAST tool (blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

Data obtained from the experiments were analyzed using descriptive statistics and a percentage bar graph was computed to analyze the relative occurrence of disease in different breeds.

Results

Breed-wide positivity

The overall positivity percentage of *Salmonella enterica* in Fayoumi breed was approximately twice as much compared to other backyard poultry breeds (Figure 2).

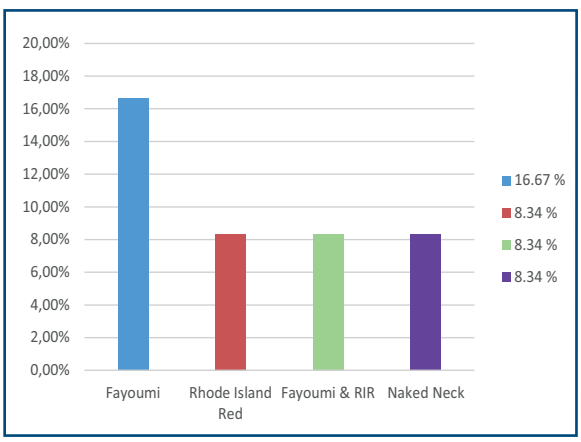

Figure 2. *Percentage bar graph showing higher positivity rate of* Salmonella enterica *in Fayoumi breed compared to other backyard breeds.*

Pathological study

A gross pathological study was conducted to get a complete picture of the ongoing disease and its pathogenesis.

Ante-mortem and postmortem examinations revealed gross and microscopic pathological changes consistent with *Salmonella enterica* infection. The ante-mortem observations of chickens indicated dehydration, emaciation, reluctance to move and the presence of bronze yellow diarrhea.

The postmortem revealed bronze discolouration and congestion of the liver.

Hepatomegaly was also observed along with focal necrosis. The spleen was enlarged, mottled, and congested.

The histopathologic evaluation of the liver revealed congestion of sinusoidal capillaries, cellular swelling (ballooning degeneration), congestion of central vein and ground-glass (granules in the cytoplasm) hepatocytes with the presence of multiple variable sized vacuoles in hepatocytes (Figures 3 and 4). Spleen also underwent congestion (Figure 5).

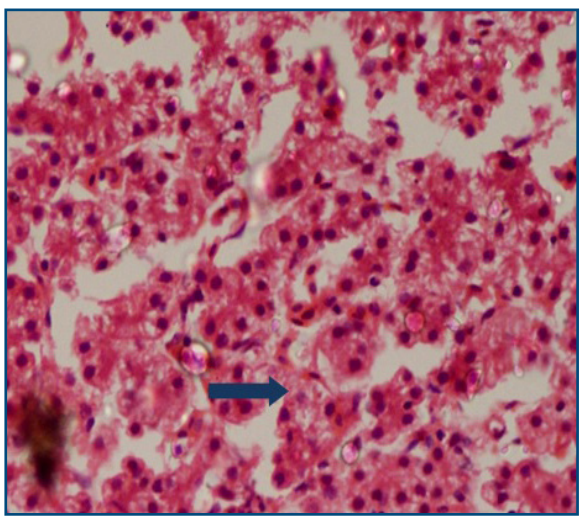

Figure 3. *Histopathology of a* Salmonella enterica *infected chicken showing congestion and disarrangement of hepatic cord. The blue arrow shows swelling (ballooning degeneration) and ground glass (granular cytoplasm) appearance of hepatocytes. (40X).*

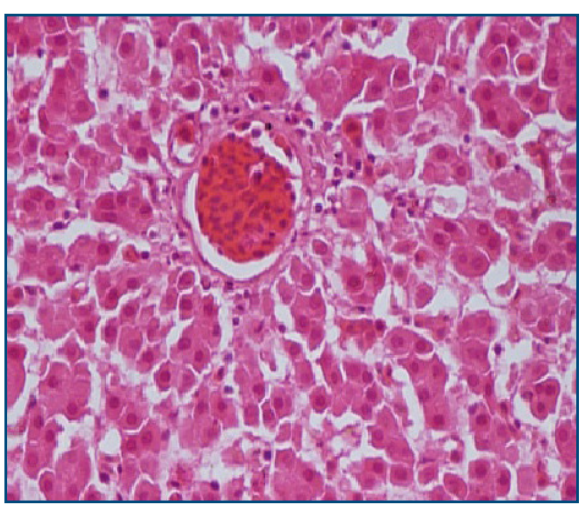

Figure 4. *Histopathology of a* Salmonella enterica *infected chicken showing congestion of central hepatic vein and ballooning degeneration of hepatocytes (40X).*

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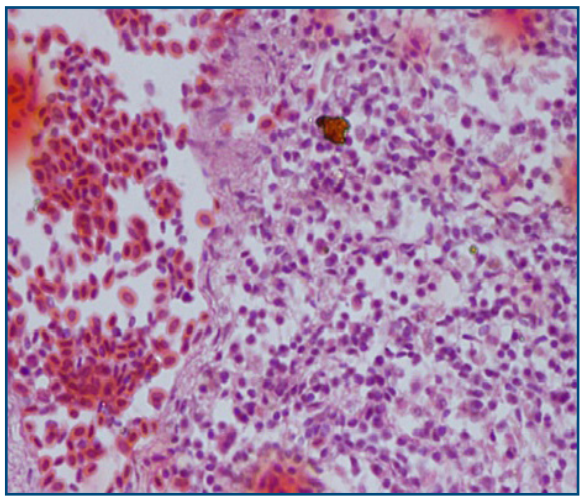

Figure 5. *Histopathology of a* Salmonella enterica *infected chicken showing congestion of splenic sinusoids (40X).*

Isolation and identification

Out of 48 samples cultured on Brilliant Green Agar (BGA), 10 showed desired growth and tested negative on Gram staining. Eight of the isolates were further characterized using biochemical tests.

Culture study

On BGA eight isolates showed light pink colonies (1-3mm) on a rose-pink background.

Morphological characterization

Gram staining revealed single and paired small pink rods indicating Gram negative bacteria.

The isolates showed hazy growth in SIM media. The morphological profile of samples isolated from backyard poultry is presented in (Table I).

Biochemical characterization

Eight isolates (Fayoumi 3, RIR 2, NN 1, and Fayoumi and RIR Crossbred 2) that were found to be non-motile, Gram negative, and revealed a growth on BGA showed variable responses to different biochemical tests.

All isolates fermented glucose but did not ferment sucrose and lactose on TSI test.

All isolates were positive for methyl red, negative for indole fermentation and Voges-Proskauer test.

The isolates that fermented dulcitol were characterized as *Salmonella enterica*, while others were considered to be *Salmonella* Pullorum.

Overall 5 samples were positive for *Salmonella enterica*, out of which 2 (16.67%) were of Fayoumi, 1 (8.34%) of RIR, 1 (8.34%) of Naked neck, and 1 (8.34%) positive sample was from the crossbred birds of Fayoumi and RIR.

A percentage bar graph of comparative positivity among four breeds is presented as Figure 2. Biochemical profile of the eight non-motile samples from backyard poultry is presented in (Table II).

Table I. *Morphological profile of samples isolated from the backyard poultry of Pakistan.*

Breed	Sample size	BGA (+ve)	Gram staining
Fayoumi	12	5	5
RIR	12	3	3
Naked Neck	12	1	1
Fayoumi & RIR crossbreds	12	1	1
Total	48	10	10

Table II. *Biochemical profile of* Salmonella enterica *isolated from backyard poultry of Pakistan.*

Breed	Non motile samples from morphological study	Carbohydrate fermentation tests			MR	VP	Indole	Dulcitol fermentation	No. of isolates
		Sucrose	Glucose	Lactose					
Fayoumi	3	-	A	-	+	-	-	A	2
		-	A	-	+	-	-	-	1
Rhode Island Red	2	-	A	-	+	-	-	A	1
		-	A	-	+	-	-	-	1
Naked Neck	1	-	A	-	+	-	-	A	1
Crossbred of Fayoumi & RIR	2	-	A	-	+	-	-	A	1
		-	A	-	+	-	-	-	1

Polymerase chain reaction

PCR targeting ratA gene for the identification of *Salmonella enterica* yielded an end product of 1047bp suggestive of a positive sample (Figure 6). All five liver samples previously positive by biochemical testing were also positive by PCR.

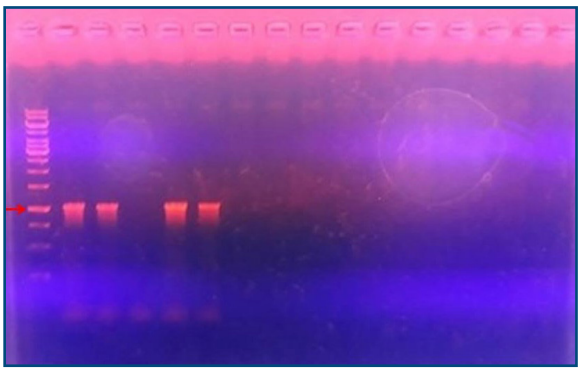

Figure 6. *Agarose gel electrophoresis on a PCR product targeting ratA gene of* Salmonella enterica *showing a band at 1047 bp. A 1 kb ladder (GeneRuler, ThermoFisher Scientific, Waltham, MA, USA) was used. A red arrow indicates 1000 bp. Lane 1: Ladder; Lane 2: Internal positive control; Lanes 3, 5, 6:* Salmonella enterica *positive samples; Lane 4: No template control (Two positive samples run in a separate batch are not shown).*

BLAST analysis

The BLAST analysis of gene sequences obtained in the present study showed 99.5% identity with the isolates of *Salmonella* Enteritidis using a standard nucleotide search tool (nblast) available online (blast.ncbi.nlm.nih.gov). The BLAST analysis revealed that the obtained sequence is similar to *Salmonella* Enteritidis isolated from human beings (SE95, SE74 and SE81) in China (GenBank accession numbers: CP050716, CP050723, CP050721) at the same time period when this study samples were collected (only three top hits in the balstn are mentioned). The sequence obtained in the present study has been deposited in the GenBank with an accession number: OM686899.

Discussion

In recent years there has been an increasing interest in people to keep backyard poultry and consume free-range chicken eggs and meat. The present study was designed to evaluate the presence of *Salmonella enterica* in four backyard poultry breeds in Pakistan.

The presence of *Salmonella enterica* in the Pakistani backyard poultry was confirmed by gross and histopathological evaluations, isolation, biochemical testing and molecular identification.

The bacterial isolate was also genetically characterized as *Salmonella enterica* subspecies enterica serovar Enteritidis.

The study revealed the presence of *Salmonella enterica* in the Pakistani backyard poultry with a percent positivity of 10.41. There is a dearth of data on the presence of *Salmonella enterica* in the backyard poultry of Pakistan.

The present study adds to the very limited published data on the presence of *Salmonella enterica* in the backyard poultry breeds of Pakistan.

Salmonellosis is a major concern in developing countries such as Pakistan as there is a lack of information and knowledge in the people regarding its sources, routes of transmission and spread, and control. Therefore, there is a need to study the presence and epidemiology of this poultry pathogen in locally relevant conditions. Salmonella control measures in most developing countries including Pakistan are not very effective owing to poor biosecurity, especially in backyard poultry settings. Moreover, Pakistan's geographical and climatic conditions support the survival and spread of the bacterium (Barrow and Neto 2011).

Since *Salmonella* Enteritidis in poultry presents a major risk for humans, the presence of *Salmonella* Enteritidis in the backyard poultry of Pakistan indicates a risk for human infection. This fact is compounded by the presence of antibiotic-resistant *Salmonella* Enteritidis in the commercial poultry of Pakistan (Siddique *et al*. 2021). Others have also confirmed the presence of multiple drug-resistant *Salmonella enterica* in the commercial poultry of Pakistan (Soomro *et al*. 2010; Wajid *et al*., 2019). Antimicrobial resistance in Salmonella has also been reported in European countries including Italy (Di Marcantonio *et al*. 2022). Growing antimicrobial resistance in *Salmonella* spp. in both poultry and humans necessitates large-scale studies exploring resistance patterns in these bacteria.

Salmonella Enteritidis control programs such as integrated farm management to prevent contamination of eggs should be implemented in Pakistan (Trampel *et al*. 2014). Globally, since the origin of most *Salmonella* Enteritidis outbreaks is infected breeding stock international trade of breeding stock should necessitate bacteria-free status as well as high biosecurity standards should be implemented to prevent local and regional dispersal (Li et al 2021). In the present study variations noted in the susceptibility of poultry breeds to *Salmonella enterica* infection is consistent with Smith (1956) who reported considerable differences in the susceptibility of various poultry breeds to Salmonella infection (Smith, 1956).

Conventional assays based on isolation and identification of *Salmonella enterica* using culture and biochemical testing are still being used as the principal mode of diagnosis and confirmation. Although effective in disease confirmation, these methods are not time-efficient as they require several days to complete. Comparatively, PCR-based methods can provide accurate and rapid diagnosis of disease outbreaks (Ma *et al*., 2014). Rapid diagnosis using PCR could help in the early detection of *Salmonella enterica* allowing government authorities and poultry farmers to take timely action to mitigate the spread of the disease. In the present study, all samples from backyard poultry that tested positive on isolation and

identification of *Salmonella enterica* were also positive by PCR suggesting very high sensitivity of PCR for detecting *Salmonella enterica*. These data suggest that PCR can be used as an alternative method that is rapid as well as accurate for the diagnosis of local isolates of *Salmonella enterica* from Pakistani backyard poultry.

When Salmonella is detected in a flock, it becomes necessary to identify the bacteria. Traditionally, isolation and biochemical identification have been used in Pakistan to differentiate *Salmonella enterica* serovars. However, since some Salmonella isolates may show atypical profiles, differentiation through biochemical testing is not always exact (Li *et al*. 1993). Therefore, in the present study PCR targeting ratA gene was performed in addition to the biochemical testing on the Salmonella isolates (Batista *et al*. 2013). The PCR-based testing identified a single product of 1047 bp suggesting that PCR can be used to identify *Salmonella enterica* from local strains of Salmonellae in Pakistan. Even though the PCR used in this study was originally developed by Batista et al. (2013) to differentiate *Salmonella* Gallinarum from *Salmonella* Pullorum, when we sequenced the 1047 bp amplicon, the obtained sequence suggested the presence of *Salmonella* Enteritidis. This is not surprising as a large-scale study analyzing Salmonella evolution has suggested that *Salmonella* Enteritidis is an ancestor to both *Salmonella* Gallinarum and *Salmonella* Pullorum (Langridge *et al*. 2015).

The 99.5% identify of *Salmonella enterica* serovar Enteritidis obtained in the present study with human isolates deposited to the GenBank at the same time when samples from this study were collected suggests an epidemiological link between human and backyard poultry Salmonella isolates. However, this is interesting that the GenBank top 3 hits to the present study *Salmonella* Enteritidis sequence were with human samples in China. Pakistan's poultry feed industry imports almost all raw materials from China and the close resemblance of backyard poultry Salmonella isolates with human isolates suggest a potential contamination of poultry feed raw materials with human feces. Contamination of feed ingredients with animal pathogens is already known and suggests a risk for the transmission of pathogens including *Salmonella enterica* via feed (Maqsood, 2012; Dee *et al*. 2014; Jones *et al*. 2020). This would imply that pre-procurement testing of raw feed ingredients for microbial pathogens could help avert incidences of such feed-borne transmission of pathogens.

The sampling for the present study was limited to the Jhelum District of the Punjab Province of Pakistan. The positivity rate of Salmonella enterica in other districts of the province and in other provinces of the country may be different and require further investigations.

In conclusion, the present study has confirmed the presence of *Salmonella enterica* in the Pakistani backyard poultry breeds. The study also suggested that PCR can be used as an alternative method for rapid diagnosis for early detection of Salmonella infection in backyard poultry in clinical samples. Integrated farm management at the country level and enhanced biosecurity at country and regional levels may need to be implemented in order to prevent the geographical dispersal of *Salmonella* Enteritidis.

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