Pakistan J. Zool., pp 1-9, 2025. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20240323132852

Molecular Detection of *Salmonella enterica* Serovar Gallinarum, Biovar Gallinarum and Biovar Pullorum from Poultry Birds (*Gallus gallus domesticus*) in Faisalabad, Pakistan

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Article Information
Received 23 March 2024
Revised 20 October 2024
Accepted 31 October 2024
Available online 23 January 2025 (early access)

Authors' Contribution
MA and AA designed the project, conceptualization, data curation, managed resources, performed major experiments and wrote original draft. MAA performed formal analysis, reviewing, formatting, editing, and financial support. AJ contributed in sample collection and performed minor experiment. MKS carried out postmortem and clinical diagnosis of poultry birds. AA and YS provided laboratory resources. IH and AA supervised overall study. All authors contributed to proof-reading and approved the final version of the manuscript.

Key words
***Salmonella*, PCR, Biovar Gallinarum, Biovar Pullorum, Poultry**

ABSTRACT

Salmonella enterica sub-species *enterica* serovar Gallinarum, biovar Gallinarum (*S.* Gallinarum/bvSG) and biovar Pullorum (*S.* Pullorum/bvSP) are the etiologic agents of fowl typhoid (FT) and pullorum disease (PD), respectively. A limited number of studies from South Asian countries had detected differentiating genes among both biovars. Molecular assay-based studies on FT and PD were also deficient from Faisalabad region of Pakistan. This study was aimed to optimize molecular detection of *S. gallinarum* and *S. pullorum* from diverse clinical samples in current laboratory settings. A total of one-hundred and thirty-four (n=134) poultry samples including; tissues (n=64), fecal (n=20), eggs (n=20) and 1-day old chicks mixed meat (n=30) were processed for the detection of *Salmonella*. Rappaport-Vassiliadis (RV) broth effectively recovered the pathogens while brilliant green agar (BGA), xylose lysine deoxycholate (XLD) agar, triple sugar iron (TSI) agar and biochemical tests by rapid kits confirmed 23.13% isolates (n=31) as *Salmonella*. By polymerase chain reaction (PCR), the *aroC* gene (encoding chorismate synthase) of *Salmonella* was detected in 13.43% isolates (n=18). The *ratA* gene (encoding region of difference; a hypothetical protein) of the pathogen confirmed 3.73% isolates (n=5) as biovar Gallinarum. A duplex PCR further differentiated FT and PD isolates by detecting *speC* gene (encoding mutated ornithin decarboxylase) in biovar Gallinarum (n=5; 3.73%) and *glgC* gene (encoding glycogen biosynthesis) in biovar Pullorum (n=3; 2.23%). The optimized PCR assay conditions of this study can be effectively used as diagnostic tool. Moreover, this study suggests the need for routine surveillance of bvSG and bvSP at the indigenous poultry production systems.

INTRODUCTION

The burden of food-borne diseases (FBD) is substantial and every year almost 1 in 10 people fall ill and 33 million of healthy life years are lost (Grace, 2023). *Salmonella* is known as one of the most important food-borne pathogens worldwide. Non-typhoidal *Salmonella* are major cause of infections caused by FBD in developed and developing countries (Elmonir *et al*., 2023). Natural reservoirs of *Salmonella* are humans, food animals (poultry, cattle, pigs), pets (cats, dogs, birds), reptiles (turtles) and rodents (rats). Multiple serovar of *Salmonella* are infecting commercial and backyard egg production units (Jajere, 2019). The bacterium can pass through entire food chain starting from animal feed, primary production, food handlers and finally to the consumers (Fàbrega and Vila, 2013). Most of the serovars cause diseases in humans while a few are host-specific and can inhabit in only one

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0030-9923/2025/0001-0001 $ 9.00/0

or a few animal species, for example, *Salmonella enterica* serovar Dublin in cattle and *Salmonella enterica* serovar Choleraesuis in pigs (Shaji *et al*., 2023). *Salmonella enterica* sub-specie *enterica* serovar Gallinarum (*S.* Gallinarum) biovars Gallinarum (*S.* Gallinarum/bvSG) and biovar Pullorum (*S.* Pullorum/bvSP) are the etiologic agents of fowl typhoid (FT) and pullorum disease (PD), respectively. Both pathogens exhibits host-specificity towards avian species primarily affecting poultry birds but turkeys, guinea fowls, parrots, sparrows and other birds can also be affected (Shivaprasad, 2000).

Possible source of *Salmonella* in poultry meat and eggs is due to cross contamination with feces (Carrasco *et al*., 2012). Although FT and PD are widely distributed among the poultry flocks across the world, but the diseases have been eradicated from commercial poultry in developed countries including Canada, Europe and United States (Kang *et al*., 2022) due to organized poultry production system. Morbidity of FT among *Salmonella* infected birds is 10%-100%, while mortality increases up to 100% due to stress in immuno-compromised birds (Batista *et al*., 2018). A meta-analysis-based study of literature has reported the global prevalence of *S.* Gallinarum as 8.54% in total samples processed during 1945 to 2021. Whereas, studies from 17 countries of Asia continent showed prevalence of *S. gallinarum* as 25.75% in Bangladesh and 19.77% in India (Zhou *et al*., 2022). Morbidity of PD among *Salmonella* infected birds is 10%-80% but there is a chance of highest mortality in bird of 2-3 weeks of age and may be up to 100% due to stress in immuno-compromised birds. *S. pullorum* occasionally causes losses among adult birds, but it causes mortality in young birds such as 20-days-old chicken (Batista *et al*., 2018). A meta-analysis-based study of literature has reported the global prevalence of *S. pullorum* as 15.79% in total samples processed during 1945 to 2021 and studies from Pakistan showed 7.33% prevalence of *S.* Pullorum (Zhou *et al*., 2022). Fowl typhoid and Pullorum disease occurrence was reported since the last decade from South Asian (SA) countries including India 69.6% incidence among broilers (Arora *et al*., 2015), outbreaks in backyard poultry (Dey *et al*., 2016), poultry production (Kumar *et al*., 2019), Bangladesh (Rani *et al*., 2022) and Bhutan (Penjor *et al*., 2023), while data from other SA countries is under-reported to date.

In vitro amplification of highly conserved genes of *Salmonella* genus by polymerase chain reaction (PCR) assay is befitting in detection of the pathogen accurately (Spickler, 2019). A duplex-PCR was developed to target *speC* gene (encoding mutated ornithin decarboxylase) in biovar Gallinarum and *glgC* gene (encoding glycogen bio-synthesis) in biovar Pullorum, simultaneously (Kang *et al*., 2011). A bunch of regions of differences (RODs) among bvSG and bvSP were identified that can be used as targets to differentiate both biovars by PCR assay. Among all those RODs, ROD-4 was a part of the *ratA* gene (a hypothetical protein), that can be used as molecular marker to differentiate bvSG from bvSP (Batista *et al*., 2013). Genome of *S.* Gallinarum has provided the breakthrough in finding the suitable gene differences to differentiate both bvSG and bvSP.

Multiple studies from different cities of Pakistan have reported *Salmonella* including prevalence of Pullorum disease based on serological testing from Kasur (Bhatti *et al*., 2013), detection of the *rfbS* gene among bvSG and bvSP isolates in raw frozen and fresh poultry meat obtained from retail markets of Quetta (Samad *et al*., 2019), sero-prevalence and pathological studies of bvSG affected birds from Faisalabad poultry farms (Shakir *et al*., 2021), pathological and immuno-histochemical findings among bvSG affected broilers of Lahore (Saleem *et al*., 2022), sero-prevalence and immunological studies of bvSP affected broilers from Faisalabad (Mahmood *et al*., 2022). However, molecular assay study reports were deficient from Faisalabad, and none of the above mentioned studies had detected differentiating genes from both biovars at molecular level. In the current study, conventional bacteriological methods such as culturing on selective and differential media (XLD, BGA etc.), biochemical testing, Gram's staining, sugar-test, are combined with molecular assay (gradient PCR, monoplex and duplex PCR, agarose gel electrophoresis), targeting, *aroC, ratA, speC* and *glgC* genes for differential identification of both biovars. The optimized PCR assay conditions can be effectively used as diagnostic tool for detection of *S. gallinarum* and *S. pullorum*.

MATERIALS AND METHODS

Samples collection

A total of one-hundred and thirty-four poultry samples (n=134) were collected from Broilers, Layers and Golden/Misri breed died due to bacterial infections at poultry farms in/around Faisalabad (Gojra) as well as from pathology diagnostic laboratory at University of Agriculture, Faisalabad (UAF). Fecal samples (n=20), eggs (n=20), tissue samples (n=64) were collected in sterile containers ensuring each sample is from different hen. In addition, mixed meat samples (n=30) were collected from 1 day old non-medicated and non-vaccinated chicks. The purpose of including a variety of samples (fecal, eggs etc) in this study was to check whether the pathogens are transferred into infected bird's faeces and eggs, or not. Samples were stored in a sunlight protected box, carrying

ice packed plastic bag (4ºC) to minimize spoilage during transportation.

Non-selective and selective enrichment

Each tissue sample was minced and 5 g of it was added in a flask containing sterilized buffered peptone water (BPW) (Oxoid, UK) and incubated at 37°C for 18-24 h. Fecal swabs were dipped into 5ml sterilized tryptic soy broth (TSB) (Oxoid, UK) and incubated at 37°C for 18-24 h. Eggs were pooled in a sterilized empty flask, stirred, and 20ml of the egg contents were added into sterilized BPW (180ml) and incubated at 37°C for 18-24 h. About 1ml of turbid growth from each sample was inoculated into sterilized Rappaport-Vassiliadis Soy (RVS) broth (Oxoid, UK) in sterilized falcon tubes for selective enrichment and incubated at 42°C for 18-24 h.

Differential and selective culturing

A loop full of turbid growth from RVS media, was streaked on MacConkey agar media (Oxoid, UK) and plates were incubated at 37°C for 18-24 h. Next day, non-lactose fermenting colonies were suspected as *Salmonella* and were chosen for further processing. Brilliant green agar (BGA) media (Oxoid, UK) was used for sub-culturing and incubated at 37°C for 18-24 h. In parallel to BGA, xylose lysine deoxycholate (XLD) agar media (Oxoid, UK) was also used and typical *Salmonella* like colonies with black centers appeared on it after incubation at 37 °C for 18-24 h.

Biochemical characterization of Salmonella *spp.*

A single well separated colony from bacterial culture on BGA or XLD agar plates was inoculated on the triple sugar iron (TSI) agar (Oxoid, UK) slants by stabbing the butt in the middle and then streaked it lightly over the slant surface area. The slants test tubes were incubated at 37°C for 18-24 h, to check sugar fermentation by *Salmonella.* Other biochemical tests of the pathogen were detected by using rapid biochemical test panel RapID™ ONE System (Thermo Fisher Scientific™), according to the manufacturer's guidelines, visible color reactions were noted and compared to the reactivity patterns available in database (ERIC® software) to interpret the results.

DNA extraction for purified DNA

The isolates were confirmed on the molecular basis by regular, nested, and duplex Polymerase chain reaction (PCR) assay. Purified *Salmonella* colonies were enriched for overnight in sterilized 5 ml TSB broth. Total genomic DNA from the bacterial culture was extracted by rapid DNA extraction kit (Thermo Fisher Scientific™) according to the manufacturer's guidelines. The purity and integrity of extracted DNA sample was estimated by using NanoDrop spectrophotometer (Thermo Fisher Scientific™).

Molecular assay for identification of Salmonella *spp.*

For the amplification of desired genes, oligonucleotide primers (Thermo Fisher Scientific™), ready to use PCR super mix (AccuPrime™ Supermix II, Invitrogen, USA) and purified DNA were further used the reaction. *Salmonella enterica* isolates were confirmed by targeting *aroC* gene (Kidgell *et al*., 2002) by monoplex PCR. The sensitivity and/or specificity of PCR was increased by applying nested PCR (Kidgell *et al*., 2002), by using the product of monoplex PCR as template. The primer sequences, thermal cycler conditions and the amplicon size of targeted gene in base-pair (bp), is shown in the Table I.

Table I. Primer sequences and thermal cycler conditions used in this study.

Target gene	Primers Sequences (5'-3')	Thermal cycler conditions (Temp. & Time)						Amplicon size (bp)	References
		Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles		
aroC	F GGCACCAGTATTGGCCTGCT R CATATGCGCCACAATGTGTTG	94ºC for 5 min.	94ºC for 45 sec.	57ºC for 1 min.	72ºC for 1.5 min.	72ºC for 7 min.	20	639	Kidgell *et al.,* 2002
aroC-N	F CCTATGAGCAGAAATACGC R GATTTCATCGCGATTCTGGC	94ºC for 5 min.	94ºC for 45 sec.	50ºC for 1 min.	72ºC for 1 min.	72ºC for 7 min.	20	460	Kidgell *et al.,* 2002
ratA	F GACGTCGCTGCCGTCGTACC R TACAGCGAACATGCGGGCGG	94ºC for 3 min.	94ºC for 1 min.	63ºC for 30 sec.	72ºC for 1 min.	72ºC for 5 min.	25	1,047 (bvSG) 243 (bvSP)	Batista *et al.,* 2013
speC	F GATCTGCTGCCAGCTCAA R GCGCCCTTTTCAAAACATA	94ºC for 5 min.	94ºC for 30 sec.	61ºC for 30 sec.	72ºC for 30 sec.	72ºC for 7 min.	25	174 & 252 (bvSG) 174 (bvSP)	Kang *et al.,* 2011
glgC	L CGGTGTACTGCCCGCTAT R CTGGGCATTGACGCAAA								

Molecular assay to differentiate bvSG and bvSP

A monoplex PCR was performed to confirm *Salmonella enterica* serovars Gallinarum biovar Gallinarum and/or biovar Pullorum by targeting *ratA* gene that produced a single product of either 1,047 bp in case of bvSG, or 243 bp in case of bvSP (Batista *et al*., 2013). A duplex PCR was performed to differentiate bvSG and bvSP by targeting *speC* and *glgC* genes simultaneously, that produced two amplicons of 174 bp and 252 bp in case of bvSG or one amplicon of 174 bp in case of bvSG (Kang *et al*., 2011). The primer sequences, thermal cycler conditions and the amplicon size of each targeted gene in base-pair (bp), is shown in the Table I.

The amplified PCR products were electrophoresed on 1.5% or 2.0% agarose gel, according to the amplicon bp size. To estimate the amplified gene bp size DNA ladder (Invitrogen) was used, gel was visualized under UV illumination system and the image was captured by gel documentation system (GelDoc-IT ™ imaging system).

RESULTS

Culturing on the differential and selective agar media results showed 47 isolates (35.07%) samples positive for typical *Salmonella* like growth. On MacConkey agar media non-lactose fermenting colorless colonies, on BGA agar media red-pinkish-white opaque colonies and on XLD agar media red colonies with black centers (H_2S production) were confirmed as typical *Salmonella* like colonies. A total of 31 isolates (23.13%) were confirmed positive for typical *Salmonella* like growth on TSI agar slants as a result of dextrose fermentation (red slant, yellow butt, blackening of the medium) and H_2S production (Fig. 1). Gram's staining results of all isolates showed gram negative rods under microscope. Biochemical tests on Remel RapID ONE identification system confirmed all of the isolates as *Salmonella*. The *aroC* primers are specific for genus *Salmonella* that amplified *aroC* gene (encoding chorismate synthase) of the pathogen, produced an amplicon of 639 bp thus confirmed 18 isolates (13.43%) as *Salmonella* by molecular assay. A nested PCR was performed using *aroC* nested primers that further confirmed *Salmonella* by generating an amplicon of 460 bp. Monoplex PCR used the *ratA* primers specific for biovars Gallinarum and Pullorum, amplified *ratA* gene (encoding region of difference; a hypothetical protein) of the pathogen and produced single product amplicon of 1,047bp thus confirmed 5 isolates (3.73%) as biovar Gallinarum (Fig. 2). None of the isolates showed an amplicon of 243bp that was expected in case of biovar Pullorum. Duplex PCR that used *speC* and *glgC* primers simultaneously, to differentiate biovar Gallinarum and biovar Pullorum. The *speC* gene (encoding mutated ornithin decarboxylase) of the pathogen was amplified and produced two amplicon of 174 bp and 252 bp thus confirmed 5 isolates (3.73%) as biovar Gallinarum. The *glgC* gene (encoding glycogen bio-synthesis) of the pathogen was amplified and produced a single amplicon of 174 bp thus confirmed 3 isolates (2.23%) as biovar Pullorum. No bvSG or bvSP (0%) was recovered from fecal samples and egg's contents (Fig. 3).

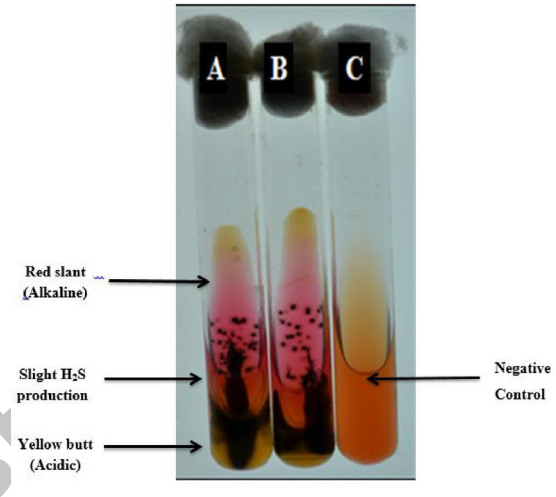

Fig. 1. Biochemical identification of *Salmonella* isolates on TSI agar slants. **A** and **B**: Typical *Salmonella* like TSI agar slant. **C**: TSI agar slant as negative control.

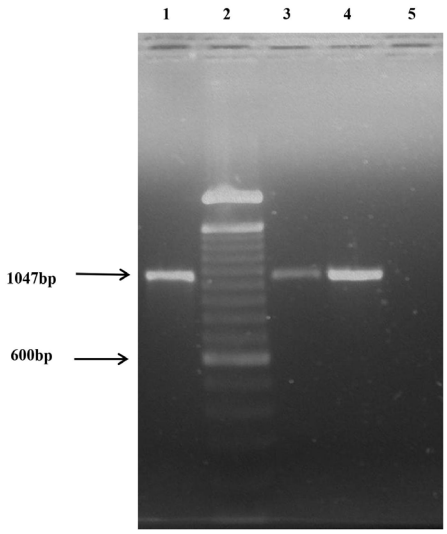

Fig. 2. Monoplex PCR targeting *ratA* gene. Lane 1, 3, 4: Amplified product of 1,047 bp of *ratA* gene fragment. Lane 2: Molecular weight marker (Invitrogen) showing fragments between 1500 bp to 100 bp in descending order. Lane 5: Negative control.

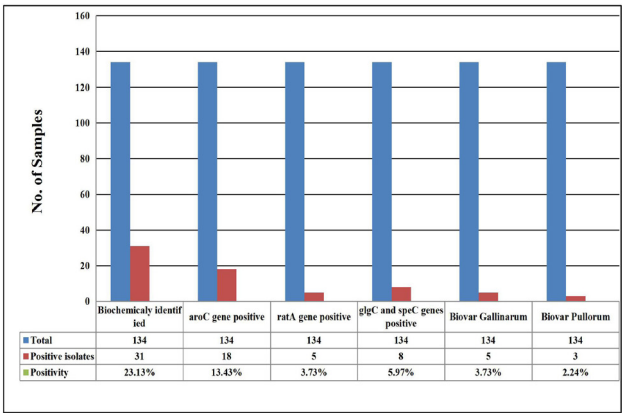

Fig. 3. Positive isolates of *Salmonella enterica* biovar Gallinarum and biovar Pullorum.

DISCUSSION

In the present study, conventional bacteriological methods for characterization of the pathogen including culturing, sugar fermentation, hydrogen sulfide (H_2S) production, biochemical characterization tests are combined with sensitive and specific molecular assay method to detect biovars. For sample collection, common signs observed postmortem lesions were similar to those described earlier (WOAH Terrestrial Manual, 2018). About 47 cultures out of 134 samples, were found positive as they showed typical S*almonella* cultural characteristics on agar media plates. The culture results were similar to the studies as reported before (Khan *et al*., 2014; Sohail *et al*., 2021). The percent positivity of the present findings is recorded as 35.07% based on conventional culture methods. The result nearly matches to the previous findings which reported 36.50% positivity of the pathogen based on culture methods, from poultry samples (Habib-ur-Rehman *et al*., 2004).

In this study, *Salmonella enterica* serovar Gallinarum (bvSG, bvSP) were not recovered from cloacal swabs and eggs. The prevalence of *Salmonella*-positive isolates in faeces and egg contents can be variable. Previous studies have reported several factors for this variability, including; sample size, season of sample collection (winter/summer etc), flock condition (free ranged/controlled shed etc.), bird's health, hygiene conditions, techniques used and many other factors (Soria *et al*., 2012). A total of 31 *Salmonella* isolates (23.13%) were obtained from tissue samples, that were further confirmed by rapid biochemical tests kit. The results based on biochemical methods are nearly equal to the previous findings, which used the same conventional and/or rapid biochemical tests for the isolation of S*almonella* spp. from chicken meat (Gast and Porter, 2019). So, the findings of this study strongly match to previous studies (Begum *et al*., 2010; Singh *et al*., 2010; Menghistu *et al*., 2011; Shahzad *et al*., 2012).

Molecular confirmation was carried out targeting *aroC* PCR assay for detection of *Salmonella* at genus level. The present findings showed 18 isolates (13.43%) positive for presence of this gene, that produced an amplified product of 639 bp, out of 31 isolates obtained by culture methods from tissue samples. The *aroC* primers have been utilized in the previous studies and are specific for *Salmonella* (Kidgell *et al*., 2002). It was attributed to the fact that irrespective of the growth potential, PCR can detect target sequences of the target cells. The sensitivity of assay was increased up to 100% by using nested *aroC* PCR primers.

S. gallinarum carries a mutation in *speC* gene encoding ornithin de-carboxylase, making the one remaining intact arginine catabolic pathway, involving arginine de-carboxylase, an essential bio-synthetic route for putrescine. The mutation in *speC* could explain the inability of *S. gallinarum* to de-carboxylate ornithine, a defining feature of this serovars. Unlike other *Salmonella* serovars, bvSG and bvSP are unable to produce glycogen. In bvSG glycogen metabolism is altered through mutations in the *glgA*, *glgB*, *glgC* genes that encodes glycogen production, while bvSP does not possess the same deletions in *glgC*, and this gene may be detected as differentiating feature of both biovars (Barrow and Neto, 2011). For differential identification of the both biovars, the duplex PCR assay targeting *speC* and *glgC* genes was performed. It successfully differentiate both biovars bvSG (3.73%) and bvSP (2.23%) in this study and produced similar results as described previously by Kang *et al*. (2011).

Previously during 2000s, other DNA based detection techniques were developed to differentiate these biovars, some of which require restriction fragment length polymorphism (RFLP) analysis after regular PCR (Park *et al*., 2001; Kisiela *et al*., 2005). The cost of extra enzymes and the requirement of further steps are evident disadvantages when correlated against the assay presented herein. Earlier studies have used an allele-specific PCR assay to differentiate the serovar Gallinarum and its both biovars based on the polymorphism of the *rfbS* gene (Desai *et al*., 2005; Shah *et al*., 2005). For that analysis, using different primer combinations, a given DNA sample must be tested two times. Furthermore, there was considerable risk of false negatives through technical failures. In contrast, the PCR assays described in this study generated amplicons of different sizes for each biovar, thus avoiding this potential problem and analysis time.

A duplex PCR assay was developed during 2011,

based on an 11-bp deletion in the *glgC* gene (a pseudo-gene in *S. gallinarum*) and a 4-bp deletion in *speC* (a pseudo-gene in both biovars). In bacterial genomes, pseudo-genes are continually created from ongoing mutational processes and are subject to degradation and removal by further accumulation of mutations. Their retention time seems to be extremely short and, even in very closely related bacteria, they tend to be deleted at a relatively rapid rate. The 793-bp difference between the biovars used in the current study occurs in *ratA*, a gene which, from the genome annotation, is not a pseudo-gene in *S. gallinarum* or *S. pullorum*. In addition, no premature stop codons were noticed in the open reading frames of *ratA* in either biovar. The *ratA* gene has also not been found to be a pseudo-gene in any other *Salmonella* serovars examined to date. The ROD located at *ratA* is thus more suitable for differentiation between *S.* Gallinarum and *S.* Pullorum than molecular markers used previously (Batista *et al*., 2013; Farhat *et al*., 2024). The PCR assay based on this gene may show a powerful tool for differentiating these two biovars when performed from isolated colonies of the *Salmonella* spp.

An overview from other countries shows that, *S. pullorum* outbreaks in adult layers are investigated by using whole genome sequencing (WGS) in China (Hu *et al*., 2019), Netherlands (Molenaar *et al*., 2023) and France (Bouquin *et al*., 2021), so far. A study from Brazil published a complete genome of a field and vaccinal strain of bvSG and compared their genomic characteristics (Chacón *et al*., 2023). While studies including advanced molecular methods for the detection of poultry isolates are limited from Pakistan, except a study on PCR-based detection of bvSG isolates from samples of Lahore poultry (Munir *et al*., 2023).

Routine surveillance of bvSG and bvSP in the poultry production system is needed. Moreover, this study suggests need of strengthening the monitoring prog for control of *Salmonella* in food production chain and promotion of national policies to reduce the emergence of drug-resistant bacterial strains. In addition, interventions such as vaccines, probiotics and natural herbs can be considered to reduce bacterial burden and spread at animal-human-environment interface. It is essential to raise awareness among all those involved in the poultry industry (farmers, poultry farm workers, technical staff, etc.) to be able to detect any outbreak quickly.

CONCLUSION

The application of PCR as a molecular assay for the rapid detection of *Salmonella* species is a promising tool and it has the potential to be applied to the diverse clinical samples as it is highly sensitive and specific test. The differential identification of the biovar Gallinarum and biovar Pullorum helps in earlier confirmation of infections and effective eradication of Fowl typhoid and Pullorum disease from the flocks respectively. The methods optimized in this study may decrease the time of diagnosis and increase the specificity and sensitivity for precise diagnosis and timely start of targeted antimicrobial therapy.

DECLARATIONS

Acknowledgments

The research work was technically supported by National Institute for Biotechnology and Genetic Engineering College, Pakistan Institute of Engineering and Applied Sciences (NIBGE-C, PIEAS), Faisalabad, Pakistan.

Funding

This study did not receive grant from any kind of funding agency (public, commercial or non-profit organization, etc).

IRB approval

The study was approved by Institutional Review Board (IRB) of the University of Lahore, Pakistan.

Ethical statement

No culling of birds or any drug intervention was required to conduct the proposed study. All the experimental procedures were conducted on the specimens collected from dead birds. All the poultry farm owners gave informed consent before taking part in the current study.

Statement of conflict of interest

The authors have declared no conflict of interest.

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