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Original Article

Molecular detection of *Salmonella* isolated from commercial chicken

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Abstract

Background: *Salmonella* in chicken, specially, the motile salmonellae, causes the food chain unsafe from farm to table and is considered a significant global threat to public health. **Aims:** The present study was carried out for molecular detection of Salmonellae in commercial poultry using PCR. **Methods:** The study was conducted for eight months, from July 2019 to February 2020, and a total of 26 poultry farms, including 15 broiler and 11-layer farms, were visited individually. Pooled faecal samples were obtained from the sheds. A total of 189 necropsy cases were examined for gastrointestinal lesions. Isolation and identification of the organism were done using microbe culture method, and the molecular characterization was performed via PCR targeting *inv*A and *ent* genes. **Results:** The prevalence of salmonellosis in the broiler and layer farms was recorded at 20.0% and 45.4%, respectively, through the traditional gold standard culture method. From 189 necropsy birds, salmonellosis was recorded at 1.58% dead cases. Molecular detection of *Salmonella* isolates by PCR targeting *inv*A gene was confirmed in 13.33% of the broiler farms and 36.3% of the layer farms. Further detection of *Salmonella enteritidis* was performed by PCR targeting *ent* gene by which 11.11% positivity was determined. **Conclusion:** This study, focused on the *Salmonella* prevalence, highlighted the zoonotic importance of the bacterium in the commercial poultry farms, which can subsequently be dispersed into the human food chain causing harmful health effects.

Key words: Molecular detection, PCR, Poultry, *Salmonella*, *S. enteritidis*

Introduction

Indian poultry industry is evolving and emerging as the world's second largest market; nevertheless, fowl salmonellosis is becoming increasingly rampant and has a significant impact on the economy as well as the future development of poultry sector. The serotypes host specific to poultry are non-motile and include *S. gallinarum*, *S. pullorum*, whereas *S. enteritidis*, and *S. typhimurium* are the motile organisms commonly reported to cause disease in both poultry and human beings. Therefore, they are of public health importance (Barrow, 2000). *Salmonella enteritidis* is recognized as a major cause of human salmonellosis worldwide, and most human salmonellosis is due to the consumption of contaminated poultry meats and by-products (Hyeon *et al*., 2021). In recent years, a shift in *Salmonella* serotypes related to poultry and poultry production has been reported in diverse geographical regions, particularly associated with the spread of certain well-adapted clones.

The intestinal disease has a major impact on the broiler industry due to economic and welfare reasons. The intestinal disease might occur due to many reasons varying from well-defined pathogens to non-specific enteritis and complex syndromes (Veen *et al*., 2017). Pathology of these diseases relies not only on the host immune status and strain of the pathogen, but also is related to the predisposing factors like age of birds, season, environmental temperature, method of rearing, and nutritional plane (Balachandran *et al*., 2013). Despite the importance of intestinal disease, determination of the cause of the disease is often difficult because many infectious and non-infectious factors may influence intestinal health (Pantin-Jackwood, 2013). Antibiotics are widely used in cattle and poultry production for a variety of reasons, including treatment and growth promotion, which has resulted in the evolution of antibiotic-resistant *Salmonella*, making *Salmonella* infection treatment increasingly difficult with each passing year (Vaez *et al*., 2020).

The present study was conducted for molecular detection of *Salmonella* bacteria in commercial poultry

in the Jabalpur region of Madhya Pradesh.

Materials and Methods

Poultry farms

The study was conducted on domestic fowl of all age groups, both sexes, and breeds. A total of 26 poultry farms including layer (11) and broiler farms (15), covering approximately 100 km radius of different areas of Jabalpur city, were included in the study.

Necropsy examination

A total of 189 necropsy cases of birds were examined at the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Jabalpur. They recorded for the presence of gastrointestinal lesions suggestive of salmonellosis. Liver and intestinal swabs were collected aseptically from the carcasses.

Isolation of organism

Pooled fecal samples were collected from poultry farms in buffered peptone water (BPW) for pre-enrichment, and samples were incubated overnight. Liver and intestinal swab samples were also collected in BPW at the time of necropsy. The samples were then inoculated into selective enrichment media (Tetrathionate (TT) broth and Rappaport Vassiliadis (RVS) Medium) and incubated at 37°C and 42°C, respectively, for 18-24 h. Organisms from the broth medium were then streaked onto selective media (XLD and BGA), and incubated at 37°C for overnight for isolation of the organism.

Latex slide agglutination test

Latex slide agglutination test was done for the confirmatory identification of presumptive *Salmonella* colonies from selective agar plates using a commercially available test kit. A bacterial colony was picked up from the selective agar plate (XLD agar) and emulsified in 20 µL of saline to produce a heavy smooth suspension. The slide was rocked gently and then observed for autoagglutination or clumping. The *Salmonella* latex reagent was gently mixed, and 20 µL of it was added next to the bacterial suspension and the slide was rocked gently and then examined for agglutination (Upadhyay *et al*., 2015). Isolates of *E. coli* were used as a negative control for the test.

Agglutination within 2 min were considered as the positive result indicating the presence of *Salmonella* in the sample. Absence of agglutination showed that *Salmonella* was not present in the test culture.

Molecular characterization of the organism via PCR

Molecular characterization of the isolates via polymerase chain reaction (PCR) was performed at the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Rewa. The isolated *Salmonella* colonies were subjected to *inv*A-based PCR assay as per the protocol developed by Scholz *et al*. (2001). A species-specific PCR was also performed for *S. enteritidis* targeting *ent* gene following Freitas *et al*. (2010) protocol.

DNA extraction

The DNA isolation from the confirmed bacterial colonies was performed as per the method described by Jofre *et al*. (2005). InstaGene mix® was mixed at moderate speed on a magnetic stirrer to maintain the matrix in suspension.

An isolated bacterial colony was picked up and resuspended in 1 ml of autoclaved water in a microfuge tube. After the centrifugation for 1 min at 13000 × g, the supernatant was removed. Then, 200 µL of Insta Gene matrix was added to the pellet, pipetted and incubated at 56°C for 20 min. Later, it was vortexed at high speed for 10 s. The tube was placed in a 100°C boiling water bath for 8 min and then vortexed at high speed for 10 s. Spin was done at 10,000-12,000 × g for 2-3 min. The prepared supernatant was stored at -20°C.

DNA extraction from positive control

The DNA for the positive control was procured from the microbial type culture collection (MTCC), Chandigarh, India. The freeze-dried culture was then inoculated and cultured on selective agar media (XLD and BGA) to check its viability. After that, DNA was isolated from pure colonies of the positive control using the previously described protocol. Distilled water was used as a negative control to check any cross contamination in the reaction.

Primers

The published oligo primers (Table 1) specific to *Salmonella* (Galan *et al*., 1992) and *S. enteritidis* (Alvarez *et al*., 2004) targeting *inv*A and *ent* genes, respectively, were synthesized at Integrated DNA Technology (IDT) Inc. and utilized in the present study.

Table 1: Primers used for *Salmonella* characterization in PCR

Primer	Sequence (5´-3´)	Product size
*inv*AF	GTGAAATTATCGCCACGTTCGGGCAA	284 bp
*inv*AR	TCATCGCACCGTCAAAGGAACC	
*ent*F	TGTGTTTTATCTGATGCAAGAGG	304 bp
*ent*R	TGAACTACGTTCGTTCTTCTGG	

The freeze-dried oligo primers were reconstituted in nuclease free water to the volume equivalent to the mass of primer (mass/µg) and left for a day at room temperature and then stored at -20°C. Primers were then further diluted in nuclease free water to give a final concentration of 10 pmol/µL for using in the reaction.

Optimization of reaction mixture and PCR

The PCR amplification of both *inv*A and *ent* genes was carried out in a final reaction volume of 25 µL. The DNA used for standardization of protocol was extracted from the procured known culture. A 25 µL-reaction

volume was used in the PCR assay initially optimized for the concentrations of the reaction components and the PCR protocol described by Galan *et al*. (1992) using 96-well thermocycler (Applied Biosystems®). The PCR product was stored at -20°C until use.

In 25 µL reaction mixture, 2 µL of target DNA along with 12.5 µL of Dream taq Green dye PCR Master Mix, 2 µL each of forward and reverse primers and 6.5 µL of molecular biology grade water were used. The PCR was carried out as per standardized cycling conditions.

Cycling conditions

The cycling conditions for *ent* gene-based PCR were carried out according to the protocol of Frietas *et al*. (2010), but the band was not clearly visible on the gel. However, better amplified product was obtained after incorporating an initial denaturation for 5 min in the final reaction programme as shown in Table 2.

Table 2: Reaction programme for PCR

Steps	Temperature and time			
	*inv*A gene		*ent* gene	
Initial denaturation	94°C for 5 min		95°C for 5 min	
Denaturation	94°C for 1 min	} 35 cycles	95°C for 2 min	} 29 cycles
Annealing	64°C for 30 s		57°C for 2.5 min	
Extension	72°C for 10 s		72°C for 2.5 min	
Final extension	72°C for 7 min		-	
Storage	4°C for ∞		4°C for ∞	

Visualization of PCR amplified product by agarose gel electrophoresis

PCR amplification was confirmed by running 8 µL of PCR product each tube on 1.5% agarose gel at a constant voltage, 100 V for 60 min in 1X TAE buffer in mini submarine gel system (GeNei). Ethidium bromide was incorporated in 40 ml of agarose gel at the rate of 5 µL of 1% solution and 3 µL of ladder was used. The amplified product was visualized as a single compact fluorescent band of expected size under UV light and documented by a gel documentation system (E gel imager, Life Technologies).

Results

Presence of organisms at poultry farms

Based on the cultural and morphological characteristics, *Salmonella* infection was confirmed in 20.0% broiler farms and 45.45% layer farms. All farms positive for *Salmonella* had larger flock sizes ranging from 8,000-10,000, suggesting that the management of the larger flocks may pose a challenge to the poultry farmers.

Gastrointestinal lesions in poultry

The prevalence of gastrointestinal lesions in poultry of Jabalpur region was totally 77.24%, and in layers and broilers were 8.90% and 91.09%, respectively. Layers exhibiting gastrointestinal lesions were also observed for reproductive lesions and showed congestion in the ovary of five birds (38.46%) The percentage and prevalence of gross gastrointestinal lesions in birds is shown in Table 3. The gastrointestinal lesions were also present more in case of broilers than layers. The Chi-square analysis for the prevalence of gastrointestinal lesions in various months showed no significant differences (χ^2 value= 1.31), while the percentage of gastrointestinal lesions were significant in layers (χ^2 value= 17.61 at 5% level (P=0.01)).

In post-mortem cases, salmonellosis was recorded 1.58% by the gold standard culture method. The lesions were observed in various organs including liver, spleen, intestine, and ovary as 71.91%, 58.21%, 68.49% and 38.46%, respectively, and liver was found to be the most affected organ followed by intestine and spleen.

Selective enrichment of *Salmonella*

The comparison of the pre-enrichment media (Tetrathionate broth and Rappaport Vassiliadis broth) showed that RVS broth gave better recovery of *Salmonella* compared to TT broth. Both broths showed colour changes after the incubation period and white precipitate formation was also noticed in the TT broth.

Isolation of pure colonies

Using microbe culture method, *Salmonella* spp. isolates were observed 2-3 mm pinkish red colonies with black centre on XLD agar along with colour change of the media showing reddish appearance. Black centre colonies were formed due to H_2S production. In BGA, the *Salmonella* isolates appeared pinkish white colonies with change of the colour of agar medium from green to pink.

Table 3: Prevalence of gastrointestinal lesions in broilers and layers

S. No.	Month	No. of P.M.		Total No. of P.M.	No. of subjects with gastrointestinal lesion		Total No. of subjects with gastrointestinal lesion	Percentage of gastrointestinal lesions		Total percentage of gastrointestinal lesions
		Layer	Broiler		Layer	Broiler		Layer	Broiler	
1	July	02	13	15	01	10	11	50.00	76.92	73.33
2	August	03	18	21	02	14	16	66.66	77.77	76.19
3	September	03	20	23	02	16	18	66.66	80.00	78.26
4	October	02	23	25	01	18	19	50.00	78.26	76.00
5	November	03	10	13	02	07	09	66.66	70.00	69.23
6	December	02	55	57	01	44	45	50.00	80.00	78.94
7	January	03	16	19	01	14	15	33.33	87.50	78.94
8	February	05	11	16	03	10	13	60.00	90.90	81.25
Total	8 months	23	166	189	13	133	146	56.52	80.12	77.24

P.M.: Post-Mortem

Confirmatory identification of presumptive *Salmonella* colonies by latex agglutination test

Weak agglutination was shown in 45.45% isolates, while 36.36% isolates presented mild agglutination, and 18.18% had strong agglutination reaction. The isolates with strong agglutination clearly showed white coloured clumps/flakes in against latex reagent. Both isolates showing strong agglutination were obtained from post-mortem cases. Similar scoring criteria for the degree of agglutination was used by Sojka *et al*. (1998).

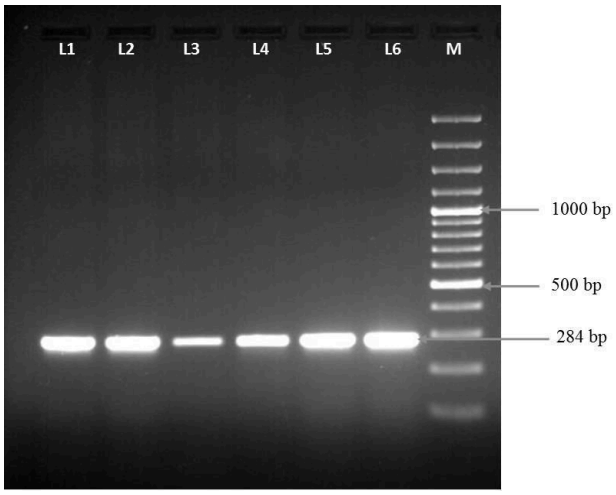

Fig. 1: Molecular characterization of *Salmonella* spp. by PCR targeting *inv*A gene with PCR amplification of 284 bp. M: GeneRuler DNA ladder, L1-L5: Positive samples, and L6: Positive control

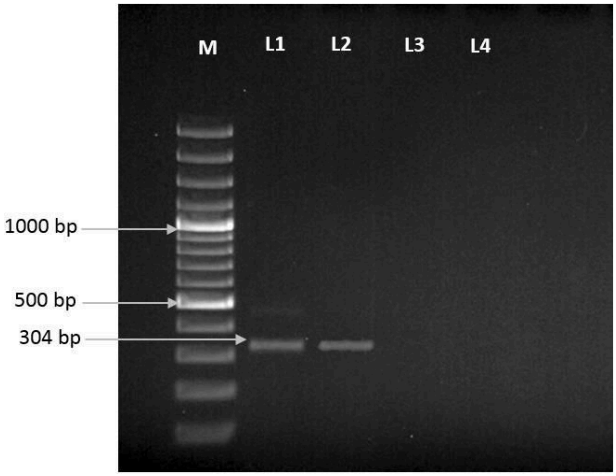

Fig. 2: Molecular characterization of *S. enteritidis* by PCR targeting *ent* gene with PCR amplification of 304 bp. M: GeneRuler DNA ladder, L1: Positive control, L2: Positive sample, L3 and L4: Negative sample

Molecular detection of *Salmonella* by PCR

Molecular detection of Salmonella spp. by genus-based PCR

Among the isolates confirmed via isolation and identification, 90.0% isolates were found positive for *Salmonella*. In positive samples, the PCR amplified product was 284 bp for *inv*A gene. Overall prevalence of *Salmonella* infection was confirmed in 13.33% and 36.36% of broiler and layer farms, respectively. In post-mortem cases, *Salmonella* was recorded in 20.5% dead birds (Fig. 1).

Molecular detection of Salmonella enterica Enteritidis

Out of the overall *Salmonella* isolates obtained in our study, the positivity of motile paratyphoid bacterium *S. enteritidis* was identified in 11.1% of isolates, while the overall prevalence of *S. enteritidis* was detected 3.84% among the twenty-six farms visited for sampling using PCR. The positive samples clearly showed 304 bp bands for *ent* gene (Fig. 2).

Discussion

Commercial poultry production has been acknowledged as one of the flourishing ventures of animal rearing in India. *Salmonella* infections of fowl may also reach the human population via contamination and mishandling of poultry products causing serious health issues. Poultry must have a healthy and functional gastrointestinal (GI) tract to maintain the excellent feed efficiency, which is required in modern poultry production, since feed constitute 66% of the total production cost (Balachandran *et al*., 2013).

All the broiler farms followed deep litter system of housing for birds, while cage system was followed in the layer farms. All sampled farms were conventional grow-out farms and broilers were reared in “all-in/all-out” housing system. In accordance with our observation, it has been reported earlier that with increased flock size of birds, *Salmonella* infection rate increases (Samanta *et al*., 2014). Our findings of gastrointestinal lesions in the liver, spleen, intestine, and ovary were comparable to those of Nazir *et al*. (2012) and Kumari *et al*. (2013).

The comparative study conducted by Rall *et al*. (2005) found RV broth more effective than TT broth. The recovery rate of *Salmonella* from XLD and BGA selective plating media using Rappaport selective enrichment media was 98.2% and 95.5%, respectively, whereas it was determined 90.0% and 64.3%, respectively using tetrathionate selective enrichment media complied with by Yhiler *et al*. (2015).

RVS broth strongly inhibit the competing organisms such as *E. coli*, *Pseudomonas* spp. and *Proteus* spp., and is more sensitive than TT broth to isolate Salmonellae (Vassiliadalis, 1983). Rappaport-Vassiliadis broth is considered superior for pre-enrichment of intestinal contents (Gorski, 2012). Our results of better recovery of organisms using RVS broth support the findings of earlier workers.

Salmonella can ferment xylose, normally decarboxylate lysine producing hydrogen sulphide (Park *et al*., 2012). Similar colony characteristics for *Salmonella* on XLD agar were observed by Nahar *et al*. (2014), Meteab and Abed (2018), and Ranjbar *et al*. (2020). Many studies have considered BGA as a selective medium to isolate *Salmonella* from poultry (Chowdhuri *et al*., 2011; Yang *et al*., 2018) with satisfactory results. Our results of agglutination agree

with Suresh *et al*. study (2019) who observed that a clearly visible agglutination reaction is seen for all *Salmonella* isolates. Similar positive agglutination reaction for *Salmonella* using latex agglutination test recorded by Gelinski *et al*. (2002).

The *inv*A gene, targeting specifically *Salmonella* genus, is suitable in the diagnostic applications using PCR assay (Rahn *et al*., 1992). According to O'Regan *et al*. (2008), *inv*A is only found in *Salmonella* species and is regarded as a golden marker in genetic characterization of *Salmonella.* In the current study, to confirm the *Salmonella* isolates, ten samples of pure colonies were subjected to PCR test targeting the *inv*A gene. Our findings were also supported by the studies of Aseel *et al*. (2011) and Salem *et al*. (2017). One *Salmonella* isolate could not be revived from the glycerol stock solution.

Overall, the prevalence of *Salmonella* infection was confirmed by PCR in 13.33% of broiler farms since one isolate could not be revived. Our result showed higher prevalence compared with Shanmugasamy *et al*. (2011) study with the prevalence of 8.3%, and lower prevalence compared to Kaushik *et al*. (2014) study with the prevalence of 23.7%.

In post-mortem cases, *Salmonella* was recorded in 20.5% of the birds that was lower compared to the study of Sharma and Das (2016) and Mshelbwala *et al*. (2018) as 43% and 86.1%, respectively, and higher compared to the studies by Nierop *et al*. (2005), Dogru *et al*. (2010), Shekhar *et al*. (2013) and Naik *et al*. (2015) as 19.2%, 8.0%, 0.94%, and 7.0%, respectively.

The infection was confirmed in 36.3% of the layer farms which were higher compared to the prevalence of 9.39% and 13.6%, reported by Bordoloi *et al.* (2017), and Soria *et al.* (2017), respectively. Based on molecular characterization by PCR, *Salmonella* was isolated from layer farms in the areas of Rampur, Amahinota, Deori, and Bargi regions in and around Jabalpur.

Culture techniques are widely accepted as the standard methods for detecting bacterial pathogens, including *Salmonella* in foodstuffs. These techniques generally take a longer time (Malorny *et al*., 2003) and are less sensitive compared to PCR-based methods (Oliveira *et al*., 2002). The use of *inv*A Gene specific PCR method in most diagnostic and research laboratories is possible. Through the molecular basis *Salmonella* identification techniques, this method is the simplest and less expensive (Shanmugasamy *et al*., 2011).

The *S. enteritidis* strains produced an amplified product of 304 bp for *ent* gene as Anjay *et al*. (2015) showed. In species-specific PCR, 11.1% of isolates found positive for the motile paratyphoid bacterium *S. enteritidis*, which was lower compared to the prevalence of 15.38% by Jinu *et al*. (2014) and Mir *et al*. (2015). The remaining isolates remained unidentified.

Out of the four confirmed areas, the motile paratyphoid bacterium *S. enteritidis* was found positive in the layer farms of Bargi region. The prevalence of *S. enteritidis* was detected as 3.84% out of the sampled farms, in the month of February in areas covering Jabalpur. While the other motile isolates remain unidentified due to time-bound research work and require further serotyping.

The reason for the introduction of *S. enteritidis* in layers may be a result of replacement birds during the period when parent breeding flocks were most likely to be infected, which led to the persistence of the bacteria in laying houses (Carrique-Mas and Davies, 2008).

A higher prevalence for *S. enteritidis* was detected by Ramya *et al*. (2012) as 19.5% and Saravanan *et al*. (2015) as 23.8%, suggesting a greater number of samplings from the farms. The prevalence of 3.1% was reported by Poppe *et al*. (1991) in the poultry environment and 2.2% was reported by Dewaele *et al*. (2012) from the caecal samples from the farms which was lower than the prevalence recorded in our study.

It is evident that the motile *Salmonella* isolates were capable of producing biofilm. Hence, they can survive in the poultry premises. Using PCR, 11.1% of isolates found positive for the motile paratyphoid bacterium *S. enteritidis* and the overall prevalence of *S. enteritidis* was detected 3.84% out of the total sampled farms. It was concluded that presence of the paratyphoid bacteria in the poultry farms is a threat of zoonotic importance since it enters the human food chain and may lead to widespread contamination in the environment.

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Conflict of interest

None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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