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Occurrence of Multi-drug Resistant Avian Salmonellae in Commercial Poultry

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ABSTRACT

Background: Salmonellosis is of great concern among the infectious diseases of poultry and has been responsible for serious economic losses to the poultry producers and a shift in *Salmonella* serotypes has been evident in recent years. Study was carried out to know the occurrence and pathology of Salmonellae infection in poultry along with their multi-drug resistance.

Methods: Samples for study were collected from 26 organized poultry farms. To determine the presence of *Salmonella* in the farms, microbial culture from fecal swabs and pooled fecal samples was carried out for isolation of bacteria. Molecular detection of *Salmonella* isolates was also performed using direct PCR. Biofilm producing ability of the bacteria was also assessed and antibiotic sensitivity test was done to detect the resistance of bacterial isolates.

Result: Salmonellosis in broiler and layer farms was recorded as 20% and 45.4% respectively and in 1.58% of the necropsy cases through microbial culture. Molecular detection of *Salmonella* isolates by PCR targeting *inv*A gene was confirmed in 13.33% broiler farms and 36.3%-layer farms. Further detection of *Salmonella* Enteritidis was performed by PCR targeting *ent* gene and 11.11% positivity was determined. Biofilm producing ability of the bacteria was found 40% using biofilm assay. Serological examination using polyvalent antisera diagnosed 27.27% isolates as motile salmonellae. During necropsy of positive cases gross lesions comparable to salmonellosis were noted in liver, intestine, spleen and ovary. Multi-drug resistant (MDR) pattern was observed with highest resistance towards oxytetracycline, streptomycin followed by amikacin, amoxicillin and enrofloxacin. The presence of multiple drug resistant *Salmonella* in chicken has rendered the food chain unsafe from farm to table and hence continuous surveillance of the disease should be encouraged.

Key words: Drug resistance, Molecular detection, Pathology, Poultry, *Salmonella*.

INTRODUCTION

Human are progressively becoming easy targets to the global health challenges, such as emerging and re-emerging infectious diseases as shown by the COVID-19 pandemic, antimicrobial resistance (AMR) and the escalating numbers of non-communicable diseases (Amuasi *et al*., 2020). Modernization of livestock farms and globalization of bird breeding trade also helps in transboundary spreading of food-borne bacteria such as *Salmonella* (Chakraborty *et al*., 2020). In recent years, problems related to *Salmonella* have increased significantly due to emergence of multi-drug resistant *Salmonella*. Increment in antimicrobial resistance interferes with the prevention and control of such organisms and represents a danger to public health (Diab *et al*., 2019).

Salmonella infection is one of the most important bacterial diseases in poultry causing heavy economic loss through mortality and reduced production (Haider *et al.,* 2004). There are relatively fewer number of reports of salmonellosis from India despite its high prevalence, which can be attributed to limited diagnostic facilities under field conditions and underreporting (Rajagopal and Mini, 2013). The contaminated faecal samples from the poultry sheds and cages can cause contamination of eggs and later chicks and hence control of bacterial infections within the poultry sheds becomes challenging (Tiwari *et al.,* 2021). *Salmonella* sp. detection in faecal samples is crucial not only for determining the aetiology but it can also aid in the elimination of the illness at the farm level (Hassan *et al*., 2020).

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Keeping the following facts in mind, the present study was conducted to determine the presence of salmonellosis in poultry and their MDR pattern in Jabalpur region of Madhya Pradesh.

MATERIALS AND METHODS

Sampling at poultry farms

The sample collection was conducted from July 2019 to February 2020 on domestic fowl of all age groups, either sex and breeds. A total of 26 poultry farms including 11-layer farms and 15 broiler farms covering different areas of Jabalpur city were included in the study. Proper data collection was performed and several aspects regarding health status of birds, biosecurity measures and other prevailing management practices.

Pathological examination

Examination of carcasses of birds (189) received at Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur from various poultry farms, was done for observing gross gastrointestinal lesions associated with salmonellosis. For histopathology, the formalin fixed tissues of liver, spleen and intestine from birds found positive for *Salmonella* following standard procedure (Slaoui and Fiette, 2011).

Isolation of organisms

Pooled faecal samples (100 g) 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. Both samples were then inoculated into Tetrathionate broth and Rappaport Vassiliadis Medium and incubated at 37°C and 42°C respectively for 18-24 hours for selective enrichment of the organism. Organisms from the broth medium were then streaked on to selective media XLD and BGA and incubated at 37°C for overnight for obtaining pure colonies of *Salmonella*.

Identification of organisms

Identification of organisms was done based on study of characteristics of colony morphology. Biochemical characterization of bacterial isolates was done using readymade biochemical kit including the motility test for differentiation of motile and non-motile salmonellae.

Molecular characterization of Salmonella isolates

Direct Polymerase Chain Reaction (PCR) was applied on collected samples using *invA*-based PCR assay for specific detection of *Salmonella* as per the protocol developed (Scholz *et al.,* 2001). Species specific PCR was performed for *S. enteritidis* targeting *ent* gene following the protocol (Freitas *et al.,* 2010). The DNA isolation from the confirmed bacterial colonies was performed using chelex resin- based DNA purification protocol as per the method described by earlier workers (Jofre *et al.,* 2005).

The published oligo primers (Table 1) specific to *Salmonella* and *S. enteritidis* targeting *inv*A and *ent* genes respectively (Galan *et al*., 1992; Alvarez *et al.,* 2004), were synthesized at Integrated DNA Technology (IDT) Inc. and utilized in present study. The cycling conditions for *ent* gene-based PCR were carried out according to the protocol of Frietas and co-workers (Freitas *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 minutes in the reaction programme (Table 2).

Biofilm assay

The biofilm producing ability of the bacteria was determined using crystal violet assay, performed as per the protocol described (Cabarkapa *et al*., 2015). For ascertainment of difference in biofilm formation, 96-well flat-bottomed polystyrene tissue culture plate was used

Table 1: Detail of primers used for PCR.

Primer	Sequence	Product size
*inv*AF	5′ GTGAAATTATCGCCACGTTCGGGCAA3′	284 bp
*inv*AR	5′ TCATCGCACCGTCAAAGGAACC 3′	
*ent*F	5′ TGTGTTTTATCTGATGCAAGAGG3′	304 bp
*ent*R	5′ TGAACTACGTTCGTTCTTCTGG3′	

Table 2: Reaction programme for PCR.

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

and quantification of biofilm formation was done through the optical densities obtained. The optical density of the wells was measured at 630 nm using an automated microtiter reader and results were presented as the median value of the six replicates.

Based on the optical densities (OD) produced by bacterial films, strains were classified into the following categories: non-biofilm producers, weak, moderate or strong biofilm producers (Stepanovic *et al*., 2003).

Identification of paratyphoid Salmonella using polyvalent antisera

A presumptive identification of motile *Salmonella* was done by using a commercially available antiserum. A small drop of antiserum (20 µl) was added on a glass slide. A small amount of positive *Salmonella* culture was then mixed with the antiserum. A positive reaction was visible as clumping on the slide.

Antibiotic sensitivity test (ABST)

The ABST was conducted by the disc diffusion method using different antimicrobials (Tendencia, 2004). Diameter of zones of inhibition was measured and antibiotics were categorized as susceptible, intermediate or resis-tant. Average of all the isolates was used to analyse the sensitivity of antibiotic toward the isolates.

RESULTS AND DISCUSSION

Based on the above-described methodologies, the following results were recorded:

Prevalence of *Salmonella* at poultry farms

All the broiler farms followed deep litter system of housing for birds while cage system was followed in the layer farms. Based on the cultural and morphological characteristics, *Salmonella* infection was confirmed in 20.0% and 45.45% of the broiler and layer farms, respectively. In post-mortem cases, it was recorded in 1.58% of dead birds.

Colony morphology

Isolates of *Salmonella* sp. were observed as 2-3 mm pinkish red colonies with black centre on XLD Agar along with change of colour of media showing reddish appearance as observed earlier (Ranjbar *et al.,* 2020). In BGA, the *Salmonella* isolates appeared as pinkish white colonies with change of the colour of agar medium from green to pink similar to previous observations of researchers (Sannat *et al.,* 2017).

Biochemical characterization of Salmonella isolates

The isolates exhibited colour change in the media present in the kit indicative of metabolic changes after 24 hours of incubation. The isolates were found negative for indole, Voges-Prausker test, urease, ONPG and lactose while they were found positive for methyl red, citrate, lysine decarboxylation as well as arabinose and trehalose utilization. However, variable reactions were observed for arginine decarboxylation, maltose and trehalose utilization in our study.

Our findings were supported by the studies conducted by Cox and Williams (1976), Howells *et al.* (2002), Murinda *et al.* (2002), Wilson (2004), Markey *et al.* (2013), Kebede *et al.* (2016), Khueankhancharoen *et al.* (2016) and Mali *et al.* (2019).

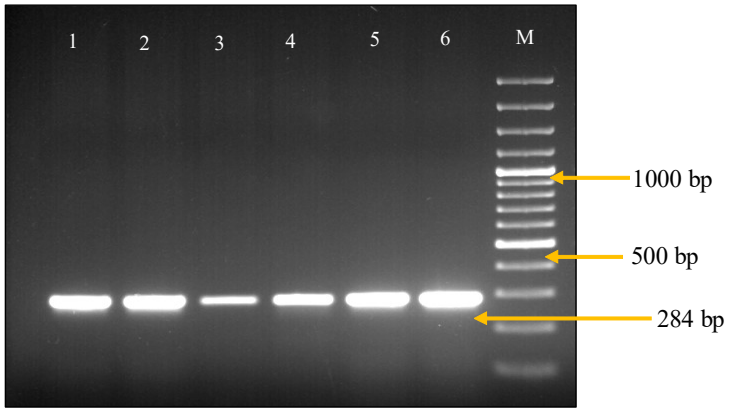
Motility test

Motile *Salmonella* were identified by the development of dark pink growth and movement of bacteria from the inoculated well no. 1 to well no. 2 in the kit. A positive result of motility for *Salmonella* sp. was indicated by the spread of the stab line as stated before (Aktar *et al.,* 2016). Out of the suspected *Salmonella* isolates, 27.27% isolates were found positive for the motility test.

Molecular detection of *Salmonella* by PCR

Molecular detection of *Salmonella* sp. by genus-based PCR

Among the isolates confirmed *via* isolation and identification, 90.0% isolates were found positive for *Salmonella.* In positive



Lane M: Gene ruler DNA ladder, Lane 1-5: positive samples and Lane 6: positive control.

Fig 1: Molecular characterization of *Salmonella* by PCR targeting *inv*A gene with PCR amplification of 284 bp.

samples, the PCR amplified product of 284 bp for *inv*A gene were clearly visible in the form of bands (Fig 1). 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.

Molecular detection of *Salmonella* enterica Enteritidis

With the help of PCR, 11.1% isolate found positive for the motile paratyphoid bacteria *Salmonella enterica* serovar Enteritidis and the overall prevalence of S. *enteritidis* was detected as 3.84% out of the total sampled farms.

Sero-grouping of motile isolates using polyvalent antisera

Salmonella somatic O poly antisera specific for motile organisms *S. enteritidis* and *S. Typhimurium* were used and among the *Salmonella* isolates obtained, 18.18% tested positive for *Salmonella* Enteritidis antisera whereas 9.09% tested positive for *Salmonella Typhimurium* antisera. A similar prevalence of 9.09% of *Salmonella typhimurium* was recorded in West Bengal (Samanta *et al.,* 2014).

Percentage positive samples of Paratyphoid *Salmonella* from broiler farms, layer farms and post-mortem cases with gastrointestinal lesions was recorded as 6.66%, 9.09% and 0.52% respectively.

Gross lesions in birds with Salmonellosis

The birds found positive for paratyphoid *Salmonella* infection were subjected to detailed necropsy examination. The birds were received with the history of anorexia, restlessness, dullness, depression and diarrhoea.

Carcasses were found to be septicemic (Fig 2A). Liver lesions comprised hepatomegaly, congestion with hemorrhagic and necrotic foci (Fig 2B) in liver. Splenomegaly along with congestion and mottling of spleen (Fig 2C) was observed. The caeca were inflamed and swollen. Severe haemorrhagic gastroenteritis and haemorrhagic typhlitis along with haemorrhagic caecal tonsils was observed. Presence of necrotic debris (caecal cores) in both the caeca (Fig 2D) was also an important finding. The lesions in ovary included in Batch-3 comprised of numerous ovarian follicles having congestion. The layers found to be *Salmonella* positive in our study were of higher age group and coagulated yolk sacs and stalk formation was not noted.

The two most consistently observed features of paratyphoid infections in mature poultry are intestinal colonization and systemic dissemination to internal organs (Gast, 2013).

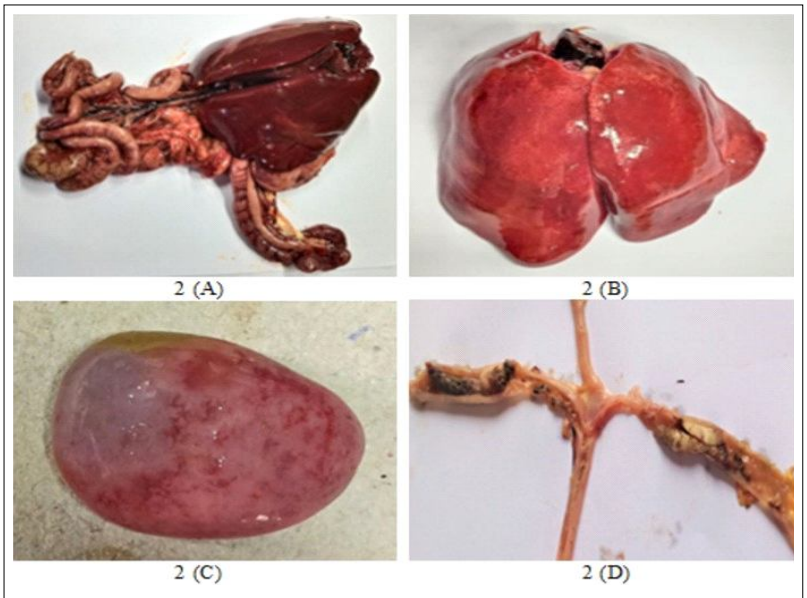
Histopathological lesions in birds with Salmonellosis

Liver

Microscopic lesions in liver included congestion, haemorrhages, hemosiderosis, congestion, dilatation of sinusoids, vacuolar degeneration, coagulative necrosis and cellular infiltration (Fig 3A). Presence of multifocal necrosis is an irreversible pathologic alteration. Kupffer cell hypertrophy was also noted. Liver was noted with maximum histopathological alterations in our study.

Spleen

Spleen showed lymphocytic follicle depletion, micro haemorrhages and fibrinoid necrosis (Fig 3B). Similar



(A) Septicaemic appearance of intestine and liver of broiler affected with salmonellosis.
(B) Necrotic foci on the liver surface of broiler affected with salmonellosis.
(C) Splenomegaly and mottling in spleen of layer bird affected with salmonellosis.
(D) Presence of necrotic debris (caecal cores) in both the caeca.

Fig 2: Gross pathology of birds affected with salmonellosis.

findings reported in previous studies (Islam *et al.,* 2006; Kumari *et al.,* 2013).

Intestine

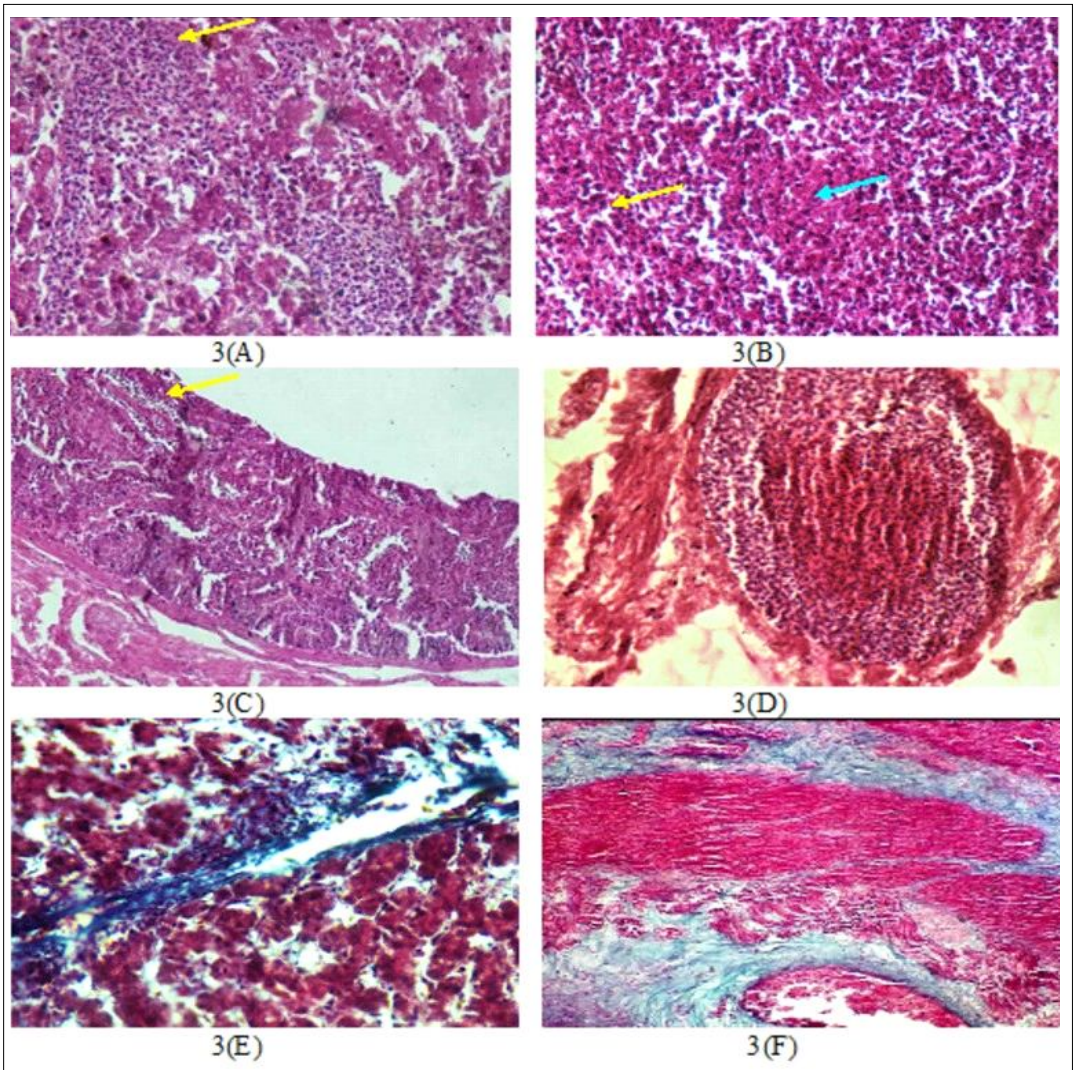
Intestine showed haemorrhages, desquamation of the epithelium and goblet cell hyperplasia. Intense cellular infiltration in the caeca and intestine was observed (Fig 3C). The findings were comparable to the microscopic lesions observed (Muna *et al.,* 2016). Haemorrhages with infiltration of mononuclear cells in the intestinal submucosa were also observed in our study similar to the previous findings (Dutta *et al.,* 2015). Special staining was done using Masson Trichrome staining in liver (Fig 3E) and intestinal sections (Fig 3F) and presence of increased amount of connective tissue was noted.

Ovary

Histopathological findings were noted in ovaries of layers included haemorrhages and huge cellular infiltration (Fig 3D).

Biofilm assay

Formation of blue coloured biofilm stained by crystal violet at the bottom of wells and on walls of the wells at air-liquid interface/pellicle biofilm was noted on visual observation.



(A) Microscopic section of liver showing extensive necrosis, punctate haemorrhage and mononuclear infiltration in portal areas (arrow). H&Ex400.

(B) Microscopic section of spleen showing fibrinoid necrosis (yellow arrow) and micro-haemorrhages (blue arrow). H&Ex400.

(C) Microscopic section of intestine showing intense cellular infiltration in the mucosa. H&Ex400.

(D) Microscopic section of ovary from bird with salmonellosis showing infiltration in the follicle. H&Ex400.

(E) Microscopic section of liver from bird with paratyphoid infection showing connective tissue. Masson's Trichromex400.

(F) Microscopic section of intestine showing collagenous connective tissue deposition. Masson's TrichromeX400.

Fig 3: Histopathological changes in birds affected with salmonellosis.

From our results, the cut-off value (OD_c) 0.174 at OD of 630 nm was used to categorize test isolates.

The result showed that 40.0% isolates possessed biofilm producing ability where 20.0% isolates were weak biofilm producers and were obtained from the necropsy cases while 20.0% isolates were moderate biofilm producers belonging to layer farms. Also, the OD value for motile salmonellae was higher than that of non-motile salmonellae in our study while 60.0% isolates were non-biofilm producers.

Antibiotic sensitivity test

Based on the antibiotic sensitivity pattern, we could elucidate that multi-drug resistance (MDR) pattern was observed in the bacteria. Out of the isolated organisms, motile *Salmonella* isolates were resistant against oxytetracycline (100%), streptomycin (66.6%) and amoxicillin (33.3%) with 66.6% non-typhoidal *Salmonella* isolates resistant to two or more than two antibiotics. Multi-drug resistance pattern was observed in 37.5% non-motile *Salmonella* isolates where maximum resistance was observed against oxytetracycline (62.5%), streptomycin (25%) followed by resistance towards enrofloxacin and amikacin (12.5% each).

Since sampling was done in commercialized broiler and layer farms, we can observe that MDR pattern has become an important challenge for the management of poultry houses. The haphazard and irrational use of antibiotics has led to the resistance of *Salmonella* towards commonly used antibiotics which is likely to aggravate with passage of time.

CONCLUSION

Salmonellosis prevalence was found to be 20.0% and 45.45% in broiler and layer farms, respectively, as established by microbial culture, with a positive rate of 1.58% in post-mortem instances. Using polyvalent antisera, the percentage of positive non-typhoidal Salmonella samples from broiler farms, layer farms and post-mortem patients with gastrointestinal lesions was 6.66%, 9.09% and 0.52%, respectively. With the use of PCR, 11.1% of the isolates tested positive for Salmonella enterica serovar Enteritidis, a motile paratyphoid bacterium. *S. enteritidis* was found to be present in 3.84% of the farms that were tested. The bacteria were discovered to be multidrug resistant (MDR), with motile paratyphoid isolates showing higher resistance than non-motile isolates. As a result, ongoing disease surveillance, including monitoring of the organisms' antibiotic resistance patterns, should be promoted.

Conflict of interest

All authors declare that they have no conflict of interest.

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