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RESEARCH ARTICLE

Prevalence and Pathology of Salmonellosis in Commercial Layer Chicken from Namakkal, India

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ABSTRACT

The prevalence of *Salmonella* species in commercial layer chicken in Namakkal poultry zone of India was investigated. Samples collected from 6572 dead birds of 85 farms during necropsy were screened for the presence of *Salmonella* species by cultural examination and two farms were positive for *Salmonella enterica* organisms and identified as *Salmonella typhimurium* in serotyping. The *S. typhimurium* isolates were amplified in polymerase chain reaction (PCR), and produced two fragment sizes *viz*. 363 bp for *kpnI* gene and 497 bp for *Pef A* gene. The prevalence of *S. typhimurium* in commercial layer farms in Namakkal area was estimated as 2.35%. Feed and fish meal was found contaminated with *S. typhimurium* and *Clostridium perfringens* and acted as source of infection in the positive flocks. The age of layer chicken of the affected farms were 24 and 30 wk with a morbidity of 3 and 4%, drop in egg production of 2 and 3% and mortality of 0.5 and 1%, respectively. On necropsy examination ovaries were congested, misshapened and oviduct serosal blood vessels were congested and mucosa contained albuminous material in 65% and cheesy plugs in 35% of the 17 positive birds. Histopathologically, decreased villi height and inflammatory changes in intestine and infiltration of inflammatory cells in ovaries and necrosis, infiltration of inflammatory cells and granulomatous reaction in the different regions of the oviduct were noticed. It was concluded that supplementation of contaminated fish meal in poultry feed could be the source of *Salmonella* infection in commercial layer flocks.

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INTRODUCTION

Indian poultry industry is one of the world largest and fastest growing industry ranking third in hen egg production (Prabakaran, 2012). Namakkal poultry zone (Tamil Nadu) occupied second position in India with a share of 20% egg production. Eggs produced from the commercial chicken flocks in this region are mainly utilized for table purpose at domestic and international level. Ninety five percent of egg export from India is constituted by Namakkal poultry zone (Anonymous, 2013). One of the main requirements of importing countries is that the table eggs should be free from zoonotic *Salmonella* species and other contagious diseases (Anonymous, 2010; Shahzad *et al*., 2012; Poole and Sheffield, 2013).

The non-motile and host specific *Salmonella gallinarum* and *Salmonella pullorum* cause fowl typhoid and pullorum disease respectively in poultry. Paratyphoid is a name given to infections of poultry caused by non host adapted motile *Salmonellae*, generally present as subclinical infection and responsible for numerous cases of food borne illness in the world. Chickens can be infected with many different serovars of paratyphoid *Salmonellae*, among these *S. Typhimurium, S. Enteritidis* and *S. Heidelberg* are worldwide in distribution with wide host range and are of major economic and public health significance (Menghistu *et al*., 2011; Huang *et al*., 2013). In addition to its impact on human health, paratyphoid in young chickens cause clinical signs of diarrhea and dehydration with a high mortality rate, whereas the infections in adult chickens do not cause significant

clinical signs or mortality, however these organisms will localize in the ovary or oviduct may result in the contamination of egg contents in naturally infected hens and constitute an insidious risk for public health (Anonymous, 2010; Jianu *et al*., 2013).

Conventional bacterial culture methods are still used most often to identify *Salmonella* and require at least 3 to 11 d (Gopalakrishnamurthy *et al.,* 2011). These methods are time consuming and labor intensive. The development of polymerase chain reaction (PCR) technology has allowed the specific amplification of particular target segments of DNA and used for the rapid detection of *Salmonella*. In the present study, primer targeting *Kpn*I enzyme gene and *pefA* gene were synthesized and used in PCR for detection of *S. Typhimurium*.

Data on the prevalence of *Salmonella* species in poultry may help to decrease the incidence of disease, health expenses and above all the confidence level of importing country will also be increased. Therefore the present study was carried out to estimate the prevalence and characterize the *Salmonella* spp. in commercial layer chicken in Namakkal poultry zone of Tamil Nadu, India, by cultural and PCR detection technique and its associated pathological changes in the affected birds.

MATERIALS AND METHODS

The prevalence of *Salmonella* species in commercial layer chicken was performed for three consecutive years (2005-2008) in 85 randomly selected commercial poultry farms located in Namakkal region of India. The dead birds (6572) were subjected to detailed postmortem examination for observation of pathomorphological changes. Tissue samples from intestine, ovary and different parts of oviduct were collected and fixed in 10% neutral buffered formalin and further processed for histopathological examination (Mashkoor *et al*., 2013).

Isolation and identification of bacteria: The liver, spleen, cloacal contents, ovary and the different segments of oviduct were aseptically collected from dead birds during necropsy examination for screening of *Salmonella* by isolation and biochemical characterization (Sujatha *et al*., 2003; Gopalakrishnamurthy *et al*., 2011). Feed, feed ingredients, water and twenty cloacal swabs from apparently healthy birds were also collected from the *Salmonella* positive flocks and subjected to *Salmonella* screening. The feed and feed ingredients were also screened for the presence of *E. coli* and *Clostridium perfringens* (Srinivasan *et al.,* 2013). Serotyping of *Salmonella* isolates was carried out at National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh, India.

Polymerase chain reaction: *Salmonella* isolates identified by conventional method was further confirmed by PCR (Cortez *et al.,* 2006) with modifications in the primer sets. Three types of oligonucleotide sequences were used as primers (*Pef* A -F 5'- TTC CAT TAT TGC ACT GGG TG-3'; *Pef*A -R 5"-AAG CCA CTG CGA AAG ATG CC-3'; *KpnI* - F 5'-AAG TTG TTC AGC TGG GTA CC-3'). The *Pef*A -R 5"-AAG CCA CTG CGA AAG ATG CC-3' was also used as reverse primer sequence for *KpnI.*

Screening for concurrent infections: Trachea, lung, spleen, caecal tonsil, kidney and oviduct collected from the *Salmonella* positive flocks were subjected to haemagglutination (HA) test for detection of Newcastle disease virus (Mohammad *et al*., 2013) infectious bronchitis virus (Villarreal, 2010) and egg drop syndrome - 76 virus (Alam *et al*., 2009). Ten serum samples collected from each *Salmonella* positive flock were analyzed for the presence of antibodies to *Mycoplasma gallisepticum* (Mg) and *Mycoplasma synoviae* (Ms) by ELISA.

RESULTS

The cultural examination of samples collected from two out of 85 farms revealed turbid growth on the tetrathionate broth, colorless colonies on MacConkey's agar and characteristic transparent pink to fuchsin colonies on brilliant green agar. The morphology of the bacterial isolates was small gram negative rods, single or paired in arrangement. The isolates were found to be motile in the hanging drop motility testing method. The organisms were further identified based on the production of alkaline slant (pink), acid butt (yellowish) with H_2S production on triple sugar iron slant. The isolates were positive for methyl red, citrate and lysine utilization and negative for the Voges Proskauer's reaction, urease, Ortho-Nitrophenyl-β-galactoside tests and indole production. The organisms fermented arabinose, maltose, sorbitol and dulcitol and produced acids and negative for lactose fermentation. Based on the cultural and biochemical reactions, the organism was identified as *Salmonella enterica*. Cultural examination of feed samples from two flocks also revealed the presence of *Salmonella enterica* organisms. To trace the source of feed contamination, the feed ingredients were subjected to bacterial examination and found that the fish meal was positive for *Salmonella enterica*. Serotyping results indicated that all isolates belonged to *S. Typhimurium*. Culture results of different samples collected from two layer flocks are presented in Table 1. In PCR assay, the *Salmonella* isolates were amplified and produced two fragment sizes *viz*. 363 bp for *kpnI* gene and 497 bp for *Pef A* gene with the sets of primers used (Fig. 1). The feed and feed ingredient samples collected from *S.* Typhimurium positive farms were also found contaminated with *Clostridium perfringens* and identified by the classical stormy clot on the sides of the tubes with excessive gas formation in skim milk medium and characteristic rough and black colonies in perfringens agar plates. Water samples did not contain pathogenic micro-organisms. Tissue samples collected for virological examination found to be negative in hemagglutination and hemagglutination inhibition test against NDV, IBV and EDS -76. All the blood serum samples tested for Mg and Ms was found to be a titer value of less than 269, hence all the serum samples were negative for Mg and Ms.

The liver of the affected birds were dark red in appearance and moderately enlarged. Gall bladder was distended with watery greenish yellow bile. Spleen and kidneys were congested and mottled in appearance. Ovaries were noticed with fully developed ovules, congested and a few follicles were misshapen. Oviduct

Table 1: Salmonella *typhimurium* serovar isolated from different samples of two layer farms

Sample type	Number of samples		Positivity (%)
	Tested	Positive	
Dead birds			
Pooled organs	30	17	56.6
Liver	30	14	46.6
Spleen	30	15	50.0
Cloacal contents	30	17	56.6
Ovary	30	16	53.3
Oviduct	30	17	56.6
Live Birds			
Cloacal swabs	40	05	12.5

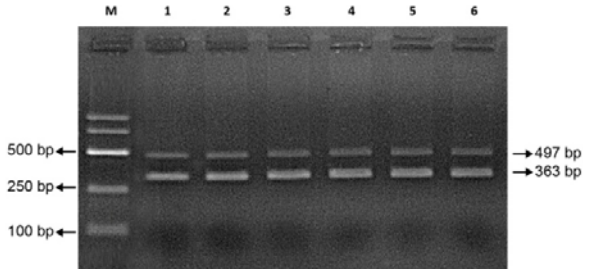

Fig. 1: Polymerase chain reaction amplification of *pefA* (497 bp) and *kpnI* (363 bp) gene from control strain and analyzed samples. Lane M: 250bp ladder. Lane 1 positive control, Lane 2: 24 wk old flock pooled organ sample, Lane 3: 30 wk old flock pooled organ, Lane 4: Cloacal swab, Lane 5: Compound feed and Lane 6: Fish meal.

serosal blood vessels were congested and the mucosa contained albuminous materials in 11 (65%) out of 17 positive birds from the two farms and cheesy plugs of varying sizes in the remaining six birds. Few birds revealed fibrinous pericarditis, extensive yellowish fibrinous peritonitis, adhesion of abdominal viscera and internal laying of soft shelled eggs.

Histopathologically, intestinal villi were decreased in height, mucosa was infiltrated with moderate amount of heterophilic granulocytes and mononuclear macrophages in the duodenal and jejunal region of the intestine. In ovary, the stroma of the follicle was expanded by congested blood vessels, fibrin and inflammatory cells (Fig. 2). Oviduct revealed necrosis and desquamation of surface epithelium, infiltration of heterophils, a few lymphocytes and plasma cells in the lamina propria and muscular layer (Fig. 3 and 4). Congestion of serosal blood vessels and multifocal expansion of the serosa by inflammatory infiltrates, and clumps of short and long bacilli were noticed on the luminal surface of all the regions of oviduct (Fig. 5). Oviduct with cheesy plugs revealed, granulomatous inflammation characterized by collection of amorphous eosinophilic material surrounded by vacuolated multinucleated giant cells, macrophages and a mixture of lymphocytes and plasma cells and layers of fibroblasts.

Among 85 farms screened for salmonellosis, two revealed the presence *S.* Typhimurium organisms with the prevalence rate of 2.35%. Age of the *Salmonella* positive flocks were 24 and 30 wk with flock capacity of 9000 and 11,000 birds, respectively. The birds showed reduction in feed intake (5 to 10 g below the expected level) in both flocks, vent pasting and general apathy in few birds. The morbidity of 3 and 4%, drop in egg production of 2 and 3% and mortality of 0.5 and 1%, were noticed in the respective age group flocks.

DISCUSSION

Identification and serotyping of *Salmonella* species in poultry is necessary for understanding and control of the associated infections. In the present study, the organisms isolated from organ samples from two farms were subjected to cultural and biochemical characterization and confirmed as *S. enterica*. The host specific *Salmonella* serovars *viz*., *S. pullorum* and *S. gallinarum* are differentiated from paratyphoid organisms by motility test, which is the fundamental basis for the identification of motile and non-motile *Salmonella* organisms (Sujatha *et al*., 2003; Gopalakrishnamurthy *et al*., 2011), whereas in the present study, the bacteria revealed progressive forward motility hence it was identified as motile *Salmonella*. The cultural prevalence in the dead birds was 17(56.6%), where as in live birds it was 5(12.5%) and concurrent with the findings of Islam *et al.* (2006).

Cultural examination of feed and fish meal samples from two flocks revealed the presence of *Salmonella enterica* and *Clostridium perfringens*. Veldman *et al.* (1995) reported that among the individual feed ingredients, animal protein supplements have higher incidence of *Salmonella* contamination. In the present study, fish meal has been used as animal protein source in both the farms. This raw ingredient was purchased from the local market by the farmers themselves and it was not subjected to laboratory tests to screen the bacterial contamination. It is generally believed that colonization of the reproductive organs is a consequence of systemic spread of *Salmonella* from the intestine (Davies and Breslin, 2001). In the present study, *Clostridium perfringens* might have enhanced the invasion of *S.* Typhimurium into the intestinal epithelial cells and colonizes various visceral organs.

The results of the serotyping revealed that all the isolates belong to the serovar *S*. Typhimurium. The dominance of one serovar over others in a particular geographical area is not uncommon (Li *et al.,* 2007), although the presence of more than one zoonotic serovar of poultry origin has been reported frequently. Purushothaman *et al.* (1996) reported that *S. typhimurium* was the commonest serotype isolated from poultry and its environment which is in accordance to the present study.

In the present investigation, detection of *S. typhimurium* was carried out by enrichment broth cultivation-PCR procedure. Cortez *et al.* (2006) used multiplex PCR targeting *pef*A gene followed by restriction digestion with enzyme *kpn*I. In the present study, instead of restriction digestion the primer targeting *Kpn*I enzyme gene was synthesized and used. PCR results were in accordance with the conventional serotyping method and corroborated with other investigators (Saravanan *et al.,* 2012).

On postmortem examination, liver, spleen and kidneys showed congestion, enlargement and mild inflammatory changes. Ovarian follicles were congested and misshapen. Oviduct serosal blood vessels were congested. Among the 17 *Salmonella* positive birds, oviduct mucosa contained albuminous materials in 11 birds and cheesy plugs of varying sizes in the remaining six. Few birds revealed fibrinous pericarditis, peritonitis, adhesion of abdominal viscera and internal laying of soft

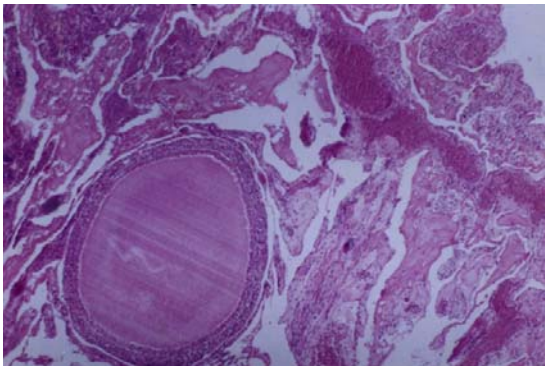

Fig 2: Ovary: Stroma of ovarian follicle was expanded by congested blood vessels, inflammatory cells and fibrin. H&E x 100.

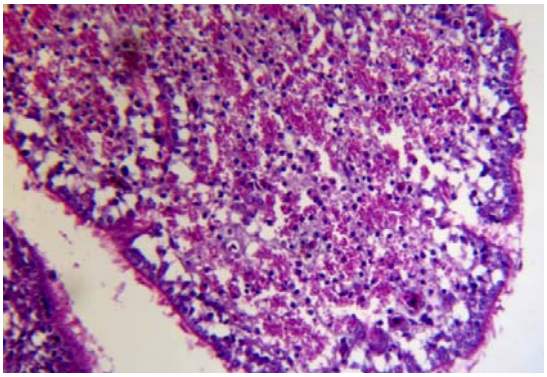

Fig. 3: Oviduct: Magnum showing desquamation of surface epithelium and infiltration of inflammatory cells. H&E x 400.

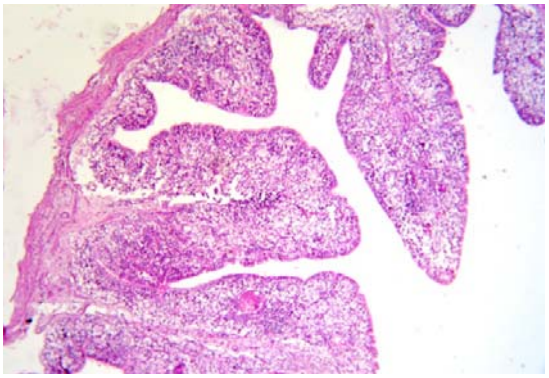

Fig. 4: Oviduct: Isthumus showing necrosis of surface epithelium and infiltration of inflammatory cells. H&E x 100.

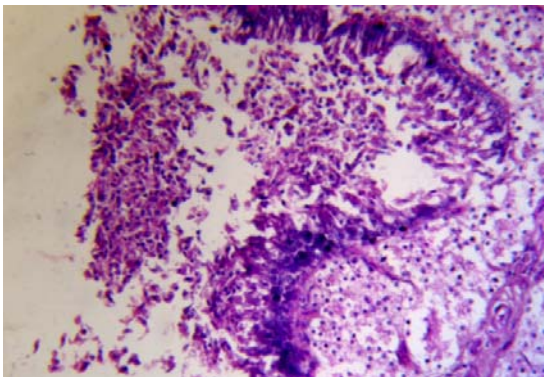

Fig. 5: Oviduct: Lumen showing accumulation of inflammatory, desquamated epithelial cells and bacterial clumps. H&E x 400.

shelled eggs (Hoop and Pospischil, 1993; Kinde *et al.,* 2000). Microscopically, in ovary infiltration of inflammatory cells and in oviduct necrosis and desquamation of surface epithelium, infiltration of inflammatory cells especially heterophils in the lamina propria and muscular layer were noticed. These changes might have been brought about by the multiplication of *Salmonella* species in the oviduct (Hoop and Pospischil, 1993) Oviduct with cheesy plugs revealed, granulomatous inflammation characterized by formation of multinucleated giant cells, surrounded by macrophages and a mixture of lymphocytes, plasma cells and fibroblast (Kinde *et al.,* 2000). In this study, both acute and chronic inflammatory changes were noticed in the oviduct, this might be related to difference in virulence of organism, genetic resistance and duration of infection.

Among 85 flocks screened for salmonellosis, two revealed the presence *S.* Typhimurium organisms with the prevalence rate of 2.35%. This was in agreement with Ibrahim *et al.* (2013) who also reported 2.1% in layer chicken. The age of the *Salmonella* positive flocks was 24 and 30 wk with flock capacity of 9000 and 11,000 birds respectively. Li *et al.* (2007) observed highest incidence during 20 to 30 wk of age. In commercial layers, the peak production occurs between the age of 25th to 50th wk, during this period the birds are subjected to considerable physiological and hormonal stress which significantly depresses immune response of layers and increases the susceptibility of *Salmonella* infection (Landers *et al.,* 2005). In both flocks, the birds showed reduction in feed intake, vent pasting and general apathy. The morbidity of 3 and 4%, drop in egg production of 2 and 3% and mortality of 0.5 and 1% respectively were noticed in 24 and 30 wk age birds. Gast and Beard (1990) also observed mortality up to 1.6% and decreased egg production in laying hens affected with paratyphoid infection.

Conclusion: Supplementation of contaminated fish meal could be the main source of *S.* Typhimurium infection in commercial layer flocks. Contamination of fish meal with *Clostridium perfringens* might have enhanced the invasion and spread of *S.* Typhimurium organisms from intestine into other visceral organs, however which require further studies for confirmation. Findings of the study stress the importance of biosecurity, particularly in respect of feed which help to reduce the chances of getting *Salmonella* infection into the flock and therefore egg contamination by these organisms.

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