Ann. Bangladesh Agric. (2020) 24 (2) : 47-57
www.doi.org/10.3329/aba.v24i2.55783

ISSN 1025-482X (Print)
2521-5477 (Online)

PATHOLOGY OF FOWL PARATYPHOID AND MOLECULAR DETECTION OF ITS PATHOGEN IN LAYER CHICKENS

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ABSTRACT

The study was aimed to ascertain the pathology of fowl paratyphoid and molecular detection of its causal agent (*Salmonella* spp) in chickens. Pathological and swab samples were collected from layers in Gazipur district, Bangladesh. For observing the gross and microscopic lesions of different organs necropsy and histopathology were done, and to isolate and identify the *Salmonella* spp*,* different bacteriological tests and Polymerase Chain Reaction (PCR) were performed. Swabs from 150 chickens showed 66% of salmonellosis. Gram's staining of isolated bacteria showed pink colored rod shaped bacilli. In biochemical tests, S*almonella* fermented dextrose, maltose, xylose, arabinose, dulcitol, mannitol except lactose and sucrose. Investigation of gross lesions at necropsy revealed hemorrhage and congestion in intestine, liver, spleen and ovaries. Necrotic foci were found in liver and spleen, and button like ulceration in cecal tonsils as well. Microscopic lesions included hemorrhage and focal necrosis in liver and spleen. Congestion and infiltrations of inflammatory cells were observed in small intestine. Ovary was hemorrhagic and there was infiltration of heterophils. Biochemically positive and isolated *Salmonella* organisms were confirmed by PCR method using invA and IE1 primers. The final results showed that a total of 91.7% *Salmonella* suspected cultures were confirmed as *Salmonella* Enteritidis.

Keywords: *Samonella*, histopathology, Gram stain*,* hemorrhage, inflammatory.

Introduction

Bangladesh is one of the highly populated country of the world, having 150 million people within the area of 147,570 square kilometers (Islam, 2014). Poultry industry in Bangladesh plays a vital role in the rural socio-economic system by contributing significantly on economic growth and simultaneously creating numerous employment opportunities. It is well known that poultry diseases are the major constraints for the development of poultry industry in Bangladesh (Karim, 2014). Major bacterial diseases which cause serious economic loss in poultry industries include

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salmonellosis, omphalitis, colibacillosis, infectious coryza and mycoplasmosis prevailing in Bangladesh. Among the diseases, salmonellosis is one of the major problems because it can be transmitted vertically. Salmonellosis has been found in all ages of poultry. It is a problem of economic concern from all production phase to marketing stage (Rahman, 2007). There are a large number of *Salmonella* serotypes that can cause a variety of diseases in different hosts.

Salmonella serotype associated with poultry reproductive tissues that have public health concern include *Salmonella enterica* subspecies *enterica* serovar Enteritidis, *Salmonella enterica* subspecies *enterica* serovar Typhimurium. Among the different serotypes, *Salmonella* Enteritidis may be better able to achieve invasion and as a consequence may be found more frequently in reproductive tissues which causes fowl paratyphoid (Mahmud, 2015). Fowl paratyphoid is generally a subclinical infection of all domestic poultry and game birds throughout the world and has also been reported in many different species of wild birds (Mark *et al*., 2008). The numerous motile members of the bacterial genus *Salmonella* are often referred to collectively as paratyphoid (PT) *salmonellae*. These organisms can infect a very wide variety of hosts (including invertebrate and vertebrate wildlife, domestic animals, and humans) to yield either asymptomatic intestinal carrier or clinical diseases (Saif, 2008).

The invA gene is functionally involved with invasion of intestinal cells of the host which codes the protein in the inner bacterial membrane (Rahn *et al*., 1992). The invA gene has been reported as a specific PCR target with important diagnostic tools for the genus *Salmonella* because it posses the unique sequence specific for the genus (Rahn *et al*., 1992; Malorny *et al*., 2003; Li *et al*., 2012). Moreover, another gene named IE1 has been reported as specific for *Salmonella* Enteritidis (Wang and Yeh, 2002; Silva *et al*., 2011; Paião *et al*., 2013). Most of the researches on natural infection of *Salmonella* in layers were done in Bangladesh using the methods of necropsy and histopathology, and isolation of *Salmonella* by culture in media, staining and sugar fermentation tests, and experimental pathogenesis, pathology and vertical transmission in chickens (Haider *et al*., 2004; Hossain *et al*., 2006; Islam *et al*., 2007; Haider, *et al*., 2008). However, very few investigations have been performed for isolation, molecular detection of *Salmonella* available in Gazipur, Bangladesh (Hosen *et al*., 2019). In order to control the PT *Salmonella* infection in poultry as well as to protect the poultry industry, histopathogical, biochemical and molecular identification need to be performed from the isolates those are endemic at Gazipur district of Bangladesh (Saha, 2012). Considering all the aspects, in the present study we have tried to observe the prevalence of PT *Salmonella* in chickens at Gazipur and to study the gross and histopathological changes in tissues in chickens as well. Moreover, we have performed the cultural, biochemical and molecular detection of the *Salmonella* Enteritidis by PCR using targeting invA and IE1 gene. Because, these two genes are allele specific for *Salmonella* Enteritidis detection.

Materials and Methods

Collection of poultry samples

A total of 150 (one hundred fifty) poultry samples including 30 sick birds on the basis of clinical signs of PT, 70 apparently healthy birds and 50 dead birds from 10 commercial layer farms were randomly collected from different areas of Gazipur district, Bangladesh during the period of September'16 to June'17. At the Post mortem examination, a systemic dissection was made along with that the gross changes in different tissues were recorded. After necropsy representative samples like lungs, liver, spleen, heart, kidney, caecal tonsils, intestine and ovary were collected in 10% buffer formalin for histopathology.

Collection of bacteriological samples

Liver, spleen, lungs and intestinal swab samples were collected from 150 chickens during necropsy. The total swab samples were 600. Aseptic cotton swabs were used and all the swabs were collected in test tubes containing 10 ml tetrathionate broth (TTB) according to methods as described previously (Haider *et al*., 2008).

Cultural media

After collection, at first, all the bacteriological samples were incubated for 24 hours in TTB. The samples were primarily cultured in Nutrient agar and then sub-cultured in the *Salmonella*-*Shigella* (SS) agar, XLD agar, Triple sugar iron (TSI) agar, Brilliant green agar (BGA) and Eosine methylene blue (EMB) agar to get desired single colony (Haider *et al*., 2008).

Morphological characterization

The presumptive colonies of suspected bacteria in various media were characterized microscopically using Gram's stain. For the separation of motile and non-motile bacteria, motility test was performed using hanging drop slide method (Cowan, 1965).

Carbohydrate fermentation test ability and biochemical test

Nine important sugars such as glucose, sucrose, lactose, mannitol, dulcitol, arabinose, inositol, xylose and maltose were used for sugar fermentation test (Cowan, 1965). The biochemical identification of PT *Salmonella* was performed using inositol and TSI agar slant (Cowan, 1965).

Processing and staining of issues

Tissue samples collected from intestine, liver, lungs, spleen, heart and ovary were fixed in 10% neutral buffered formalin and further processed for histopathological staining with hematoxylin and eosin stains (Mashkoor *et al*., 2013). Photomicrography of stained tissues was taken using photomicrographic camera (ZEISS AxioCam ERc5s).

Molecular identification of presumptive isolates

From the isolated pure culture the genomic DNA of PT *Salmonella* was extracted using DNA extracting kits (Promega Corp. Madison, WI, USA) (Haider *et al*., 2008). Extracted DNA was amplified with using primers invA and IE1 (Table 1) targeting for the gene of *Salmonella* Spp. using commercial PCR kits in gene amplification PCR system 9600 Thermocycler (eppendorf, Germany) (Silva *et al*., 2011; Shanmugasamy *et al*., 2011). The thermal profile of PCR for InvA gene was 94°C for 5 minutes (initial denaturation), 94°C, for 20 second (denaturation), 50°C for 30 second (annealing), 72°C for 30 second (elongation) and 72°C for 7 minutes (final

Table 1. Primers were used for the detection of the *Salmonella* spp

Primers	Length	Primer sequence (5′-3′)	Amplification products (bp)
invA(F)	26	GTGAAATTATCGCCACGTTCGGGCAA	284
invA(R)	22	TCATCGCACCGTCAAAGGAACC	
IE1(F)	20	AGT GCC ATA CTT TTA ATG AC	316
IE1 (R)	19	ACT ATG TCG ATA CGG TGG G	

extension), and the holding temperature was 4°C. Separation of amplified products were done by electrophoreses on 1.5% agarose gel containing 5µg per ml ethidium bromide with a 100 bp ladder as molecular weight marker (Haider *et al*., 2008).

Results and Discussion

In this study, collected chickens were subjected to bacteriological isolation and identification, gross and histopathological study of identified cases. One hundred and fifty samples were collected from 10 different layer farms of Gazipur district.

Prevalence of isolated and identified PT *Salmonella*

Among 150 chickens' samples, 99 were found PT *Salmonella* positive based on colony characters on different agar media and sugar fermentation tests. So, the prevalence of salmonellosis in this study was 66%. The prevalence rates in different organs of chickens were found different (Table 2). The highest prevalence was found in intestinal swabs followed by liver, spleen and lungs. The prevalence of salmonellosis detected in this study is similar to the findings of other researcher (Islam *et al*., 2016). Rahman *et al*., 2011 reported that the overall seropositive prevalence of *Salmonella* was 46.2% in Birgonj upazila, Dinajpur district in Bangladesh. The prevalence rate is slightly higher in this study than that of the findings of Rahman *et al*., 2011. Densely populated poultry farming in this experimental area could be the major cause of higher prevalence of salmonellosis which was supported by Donado-Godoy *et al*., 2012. Donado-Godoy *et al*., 2012 reported that *Salmonella* was isolated from 41% of farms and 65% of the 315 chicken houses sampled in Colombia.

Colony characters in cultural media

Isolated organisms were formed round, white dew drop like colonies on nutrient agar (Fig. 1a). The organisms were formed round, raised, transparent single colonies with black centers on SS agar (Fig. 1b). On XLD agar, PT *Salmonella was* produced black colonies

Table 2. Cultural prevalence of PT *Salmonella* of collected samples in different organs

Organs & site of swabs	Total swabs	Positive	Cultural Prevalence %
Liver	150	45	30
Spleen	150	35	23.33
Lungs	150	15	10
Intestine	150	75	50

(Fig. 1c). Black colonies were found on TSI medium (Fig. 1d). Red to pink white colonies was surrounded by brilliant red zones formed on BGA media (Fig. 1e). Pink colonies were showed by PT *Salmonella* in EMB agar (Fig. 1f). In the present study the colony characters of *Salmonella,* the production of hydrogen sulfide gas with black colonies on SS agar and TSI agar are similar to the results of other authors (Hossain *et al*., 2008; Saha *et al*., 2012; Lujain *et al*., 2016).

Morphological characterization

All the isolates were showed Gram's negative, rod shaped pink color bacilli with Gram's staining which was similar to the findings of other authors (Haider *et al*., 2004; Rahman *et al*., 2011; Lujain *et al*., 2016). Organisms were found motile when examined under microscope with hanging drop slide preparation that confirmed as PT *Salmonella*. This finding was supported by others (Haider *et al*., 2004).

Biochemical tests

A total of 170 isolates were purified and tested for biochemical identification. The isolated organisms fermented inositol, dextrose, maltose, xylose, mannitol, arabinose and dulcitol but did not ferment the sucrose and lactose. In TSI agar slant the organisms produced H_2S gas. The similar sugar fermentation tests were reported previously (Haider *et al*., 2004; Ahmed *et al*., 2008).

Gross lesions

Liver was congested and there was focal necrosis (Fig. 2a). Button like ulceration was

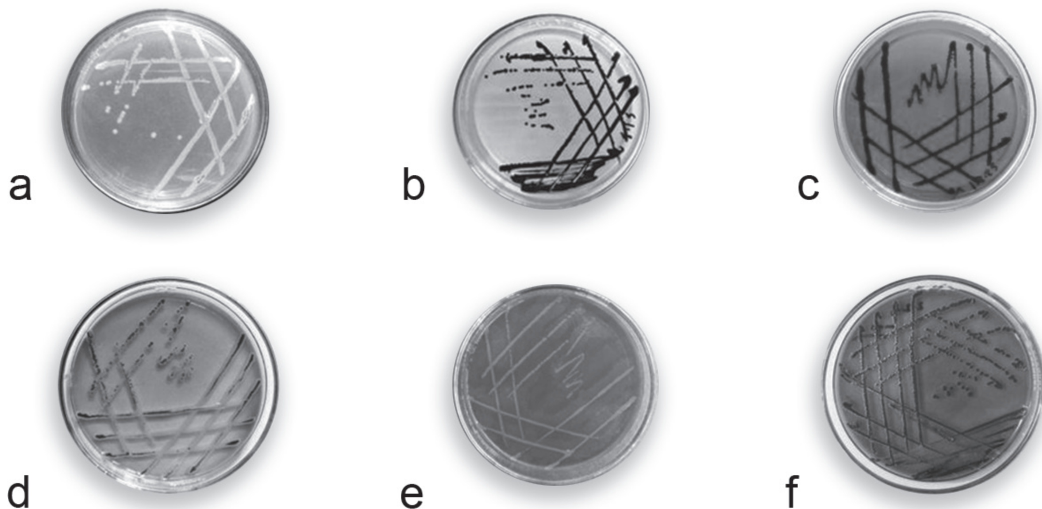

Fig. 1. a. Colonies of isolated *Salmonella* on Nutrient agar plate. b. Organism formed round, raised, transparent single colonies with black centers on SS agar. c. *Salmonella* produced black colonies on XLD agar plate. d. Isolated *Salmonella* produced black colonies on TSI medium. e. Colonies are small, opaque, pink or white on Brilliant Green (BG) agar. f. Pink color colonies of *Salmonella spp.* are on EMB agar.

recorded in caecal tonsils (Fig. 2b). Petechial hemorrhages were found in the spleen (Fig. 2c) and base of the heart. The ova were hemorrhagic, deformed and cystic (Fig. 2d). Profuse hemorrhage was found in intestine (Fig. 2e). Petechial hemorrhages were also found in the kidneys (Fig. 2f). In this study, grossly the liver and spleen samples were infected by *Salmonella* spp showed congestion with focal necrosis which was similar to the findings of other authors (Hoop *et al*., 1993; Majid *et al*., 2000; Rahman *et al*., 2011; Nazir *et al*., 2012; Saha *et al*., 2016). Profuse hemorrhage and congestion in the intestine which were correlated to the findings of other authors (Hoop *et al*., 1993; Majid *et al*., 2000; Nazir *et al*., 2012). The ovarian follicles were found congested and misshapen which had been reported earlier (Majid *et al*., 2000; Ahmed *et al*., 2008; Nazir *et al*., 2012).

Microscopic Lesions

The lesions of small intestine included severe congestion and infiltration of inflammatory cells (Fig. 3a). The prepared histopathological slide of liver section showed vascular congestion, multifocal degeneration and necrosis of hepatocytes, and infiltration of heterophils and nodular lesions with infiltration of macrophages (Fig. 3b and Fig. 3c). Section of spleen showed severe congestion and

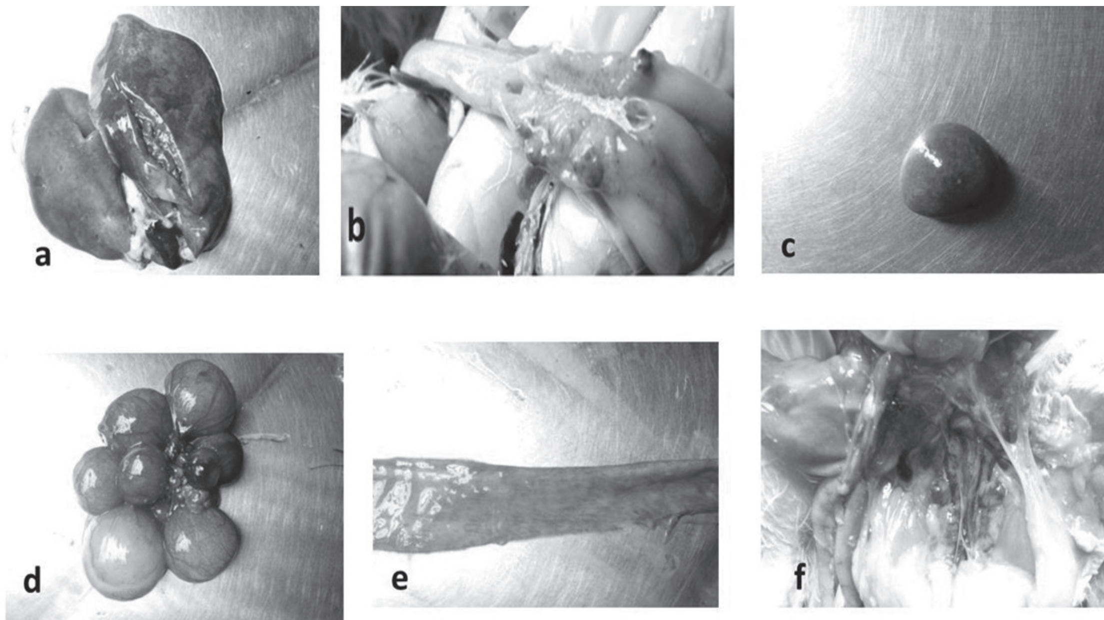

Fig. 2. a. Congested liver with focal necrosis of *Salmonella* infected chickens. b. Caecal tonsil showing button like ulceration in case of *Salmonella* infected chickens. c. Spleen of *Salmonella* infected chickens showing petechial hemorrhage and focal necrosis. d. Ova are shown hemorrhagic, deformed and cystic in *Salmonella* infected chickens. e. *Salmonella* infected chicken's small intestine showing profuse hemorrhage and congestion. f. *Salmonella* infected chickens kidneys showing petechial hemorrhage.

focal necrosis (Fig. 3d). Pulmonary lesions described that there was congestion in blood vessels and sero-fibrinous exudation in lungs (Fig. 3e). The ovary showed congestion and infiltration of inflammatory cells (Fig. 3f). The section of heart showed necrosis of the myofibers and few infiltrations of heterophils (Fig. 3g). Similar histopathogical lesions were also previously reported by others (Majid *et al*., 2000; Haider *et al*., 2004; Hossain *et al*., 2006; Ahmed *et al*., 2008; Rahman *et al*., 2011; Nazir *et al*., 2012).

Molecular Characterization by PCR

From the 170 biochemically confirmed PT positive samples, 12 presumptive salmonella were selected randomly for molecular identification. A PCR product of 284 bp was successfully amplified with PCR which is a fragment of invA gene specific for all members of *Salmonella* species. 100 bp DNA marker was used as a molecular weight marker. The band size detected in 11 of the presumptive *Salmonella* isolates (91.7%) and analyzed by agarose gel electrophoresis (Fig. 4). Again

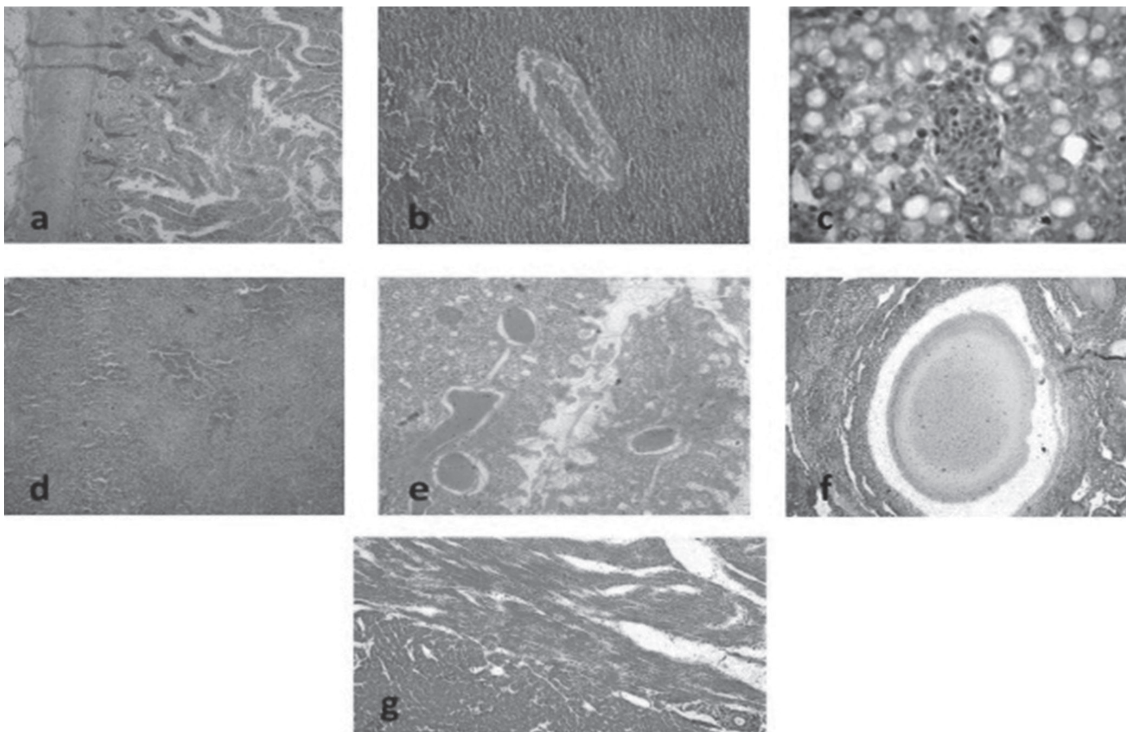

Fig. 3. a. The lesions of small intestine included severe congestion and infiltration of inflammatory cells, (H & E, X 100). b. Section of liver section showed vascular congestion, multifocal degeneration and necrosis of hepatocytes and infiltration of heterophils, (H & E, X100). c. Section of liver is showing RBC in central veins, (H & E, X 400). d. Section of spleen showed severe congestion and focal necrosis, (H & E, X100). e. Pulmonary lesions described that there was congestion in blood vessels and sero-fibrinous exudation in lungs, (H & E, X 100). f. Section of ovary shows congestion and infiltration of inflammatory cells (H & E, X 100). g. Section of heart showing necrosis of the myofibers and few infiltrations of heterophils, (H & E, X 100).

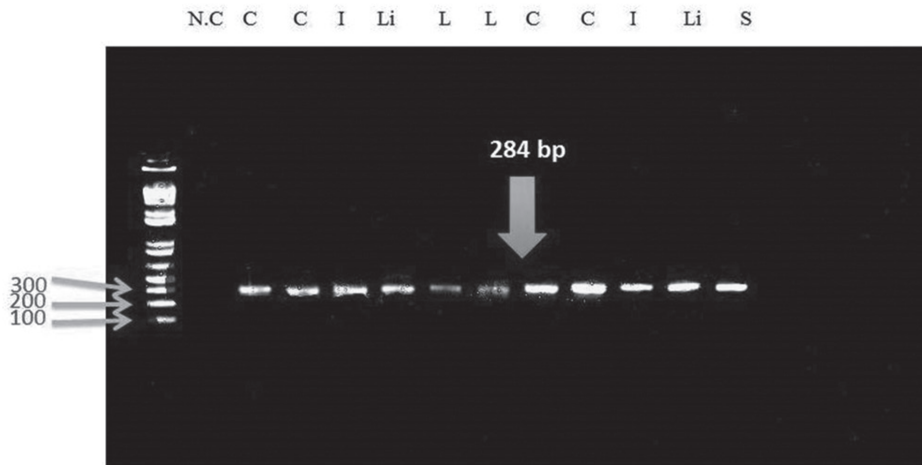

Fig. 4. Agarose gel electrophoresis for amplification of *inv*A gene (284bp) of *Salmonella spp.* (Legends- C = Cloacal swabs, L = Lungs swabs, Li = Liver swabs, I = Intestinal swabs, S = Spleen swabs, N.C. = Negative Control).

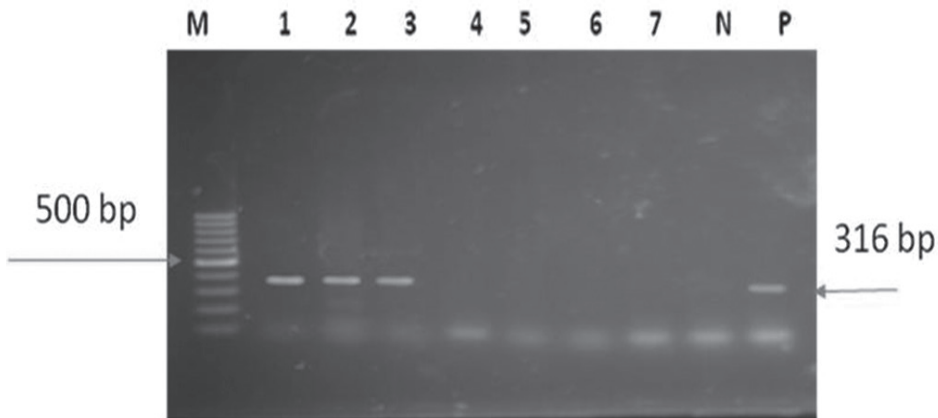

Fig. 5. Amplification of IE-1 gene (316 bp specific genomic primer; Lane M: 100 bp DNA ladder, Lane P: positive control Lane N: Negative control, Lane 1-3 DNA templates extracted from culture positive (Positive control was taken from Department of Microbiology and Public Health, BSMRAU, Gazipur).

all the invA positive samples were positive for the IE-1 gene (band size 316 bp) (Fig. 5) which revealed the isolates were *Salmonella* Enteritidis. In recent study, 91.7% samples were confirmed as *Salmonella spp* by PCR which was confirmed earlier by biochemical tests. The percentage samples were lower than that of other finding (Malorny *et al.*, 2008). It may be due to the failure of DNA extraction from the isolates. Primers invA is a rapid, sensitive, and definite for the detection of *Salmonella* spp in many clinical samples (Lampel *et al.*, 2000). Shanmugasamy *et al.*, 2011 supported the ability of these

specific primer sets to confirm the isolates as *Salmonella* spp. In this study, all the *Salmonella* spp. were identified as *Salmonella* Enteritidis when specific IE1 primer was used which suggests that 100% specificity of IE1 gene in case of detection of *Salmonella* Enteritidis. This finding are in agreements with other studies (Wang and Yeh, 2002; Silva *et al*., 2011).

Although Gazipur is considered as the prime zone for poultry industry in Bangladesh. There is no such satisfying research work on PT Salmonella. The prevalence of PT *Salmonella* which is not only important for producing hazard free poultry products but it has also zoonotic importance. Our research work will help to identify the PT *Salmonella* at farmer level with minimum lab facilities by rapid sensitive PCR, cultural and biochemical methods. It will be helpful for sustainable poultry production. Molecular detection will also help the researchers who are intended to do further research on *Salmonella.*

Conclusion

Fowl paratyphoid is generally a subclinical infection of all domestic poultry and game birds. *Salmonella* Enteritidis is one of the many causal agents of this disease which has zoonotic importance. The histopathology of fowl paratyphoid, isolation and molecular detection of its causal agent as *Salmonella spp.* were conducted in this study. The prevalence of the salmonellosis was found 66%. The gross and histopathological lesions were shown the severity of paratyphoid infections in chickens. The severity may be enhanced by the combination of other diseases like collibacillosis, mycoplasmosis and mycotoxicosis in the densely poultry populated area. Besides, these PT *Salmonella* can cause huge public health hazards in experimental area.

Acknowledgements

This research work was funded by Research Management Wing (RMW) and University Grant Commission, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur.

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