

## PATHOLOGY OF FOWL TYPHOID AND MOLECULAR DETECTION OF ITS PATHOGEN

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### Abstract

*Salmonellae* are important group of pathogens responsible for human and animal diseases. The present study was undertaken with the aim to study pathology of fowl typhoid caused by *Salmonella enterica* subsp. *enteric* serovar Gallinarum and to identify *Salmonella* serovars by polymerase chain reaction (PCR) based molecular method isolated from commercial layer, broiler and sonali chickens of Gazipur district, Bangladesh. A total of 150 cloacal, intestinal and liver swab samples were collected in sterile nutrient and tetrathionate broth from apparently healthy, sick and dead chicken the necropsy. Organ samples were collected in 10% buffered neutral formalin. The collected tissues were fixed, processed, sectioned, stained with hematoxylin and eosin (H&E) and examined at low and high power microscopic fields. Grossly, the liver appeared larger and hemorrhagic with focal necrosis. Catarrhal inflammation on intestinal mucosa was seen. The ova were deformed, discolored and cystic. Microscopically, focal necroses with the infiltration of mononuclear cells were seen with congestion of the central vein. Spleen showed severe depletion of lymphoid cells in white pulp along with reticuloendothelial cell hyperplasia. The section of ovary showed deformed ova with hemorrhages. Samples were subjected to various cultural, biochemical, and molecular examinations and the prevalence was identified 28% cases. Isolated bacteria appeared gram (-)ve, and arranged in short chain. PCR was performed targeting *invA* gene of *Salmonella* Gallinarum and amplified 184-bp fragment of the isolates confirmed specific infectivity.

**Keywords:** Fowl typhoid, chickens, *Salmonella*, pathology, molecular detection.

### Introduction

Poultry is an important component in livestock sector in Bangladesh. In many countries poultry is the principal source of economics and high quality human foods. However, it is a highly sensitive and risk oriented venture. It plays a significant role in poverty alleviation and economic development of Bangladesh. Approximately 70.4% of total animal protein supplied in the country is contributed by

poultry meat (Rahman *et al.*, 2017). To satisfy market demands for poultry meat and eggs by the mostly urban, municipal and rural populations, commercial layer farming with high yielding strains of chickens has expanded rapidly in different areas in Bangladesh. However due to this disease, the producers are facing numerous problems in farm operations and management (Arbelot *et al.*, 1997).

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Poultry is essential not only for the national economy of Bangladesh but also the welfare of human beings. Several constraints such as the diseases, poor husbandry, low productivity and high price of feed affect the optimal performance of this industry in Bangladesh (Haque *et al.*, 1991; Rashid *et al.*, 2013).

Fowl typhoid (FT) is an acute infectious enteritis (Okwori *et al.*, 2007) causing heavy mortality in growers or adult chickens although chicks can be affected (Jordan and Pattison, 1992). It is caused by the bacterium *Salmonella enteric* serovars Gallinarum (Jordan and Pattison, 1992 and Aiello, 1998), a member of the family enterobacteriaceae which is widely distributed throughout the world (Roa, 2000). *Salmonella enteric* serovars Gallinarum is highly adapted and seldom causes significant problems in hosts other than chickens, turkeys and pheasants (Jordan and Pattison, 1992; Aiello, 1998; Mdegela *et al.*, 2000; Okwori *et al.*, 2007).

The disease occurs sporadically or enzootically in most countries in the world including Bangladesh. FT losses often begin at hatching time and losses continue to laying age (Shivaprasad, 1997). *Salmonella*. Gallinarum is very important in poultry health because they are responsible for massive destruction of poultry (Gast, 1997). FT seriously threatened the poultry industry in the early 1900s due to widespread outbreaks accompanied by high mortality (Shivaprasad, 2000). Fowl typhoid is one of the major constraints of poultry industry in Bangladesh (Das *et al.*, 2005). The disease is considered as OIE, list B disease (Calnek *et al.*, 1997). Considering the above facts, the present study was designed to study

the clinical signs, gross and histopathological lesions of fowl typhoid along with isolation and identification of *Salmonella* organism from commercial chickens by biochemical and PCR-based methods.

### **Materials and Methods**

In this study a total of 50 cloacal swab samples from commercial broiler/layer chickens, 50 liver swab samples and 50 intestinal samples from apparently sick or dead chickens were subjected to bacteriological isolation, identification, histopathological study and molecular diagnosis of *Salmonella* Gallinarum. The study was performed at the Department of Pathobiology at Bangabadhu Sheikh Mujibur Rahman Agricultural University (BSMRAU) and National Institute of Biotechnology (NIB), Savar, Dhaka.

### **Collection of samples**

Dead and diseased chickens suspected to bacterial infection were collected from different commercial layer and broiler farms of Gazipur district. Samples were collected from the same flock along with the necropsy study. Using septic cotton swabs, all the cloacal samples were collected in test tubes containing 10 ml tetrathionate broth (TTB) according to methods described elsewhere (Haider *et al.*, 2008).

### **Cultural and biochemical tests**

All the bacteriological samples were incubated for 24 hours in TTB. Then, all the samples were primarily cultured in Nutrient agar and then subcultured on the *Salmonella-Shigella* (SS) agar, Triple sugar iron (TSI) agar, Brilliant green agar (BGA), Eosine

methylene blue (EMB) agar (Haider *et al.*, 2008). The presumptive colonies of different bacteria in various media were characterized microscopically using Gram's stain. Five basic sugars such as glucose, sucrose, lactose, mannitol and maltose were used for sugar fermentation test (Haider *et al.*, 2008). Motility test were performed for separation of motile and non-motile bacteria according to the method described elsewhere (Haider *et al.*, 2008).

### **Histological study of tissue samples**

Tissue samples collected from intestine, liver, lungs, heart, ovary and different other organs were collected and fixed in 10% neutral buffered formalin and further processed for histopathological examination (Mashkoo *et al.*, 2013).

### **Photomicrography**

Photomicrography was taken using photo micrographic camera (ZEISS AxioCamERc5s) facilitated by Department of Gynecology, Obstetrics and Reproductive Health, BSMRAU.

### **Detection of Salmonella Gallinarum by Polymerase chain reaction (PCR)**

From liver, lungs and other tissue samples the genomic DNA of Salmonella Gallinarum were extracted using DNA extracting kits (Promega Corp. Madison, WI, USA). (Haider *et al.*, 2009). Extracted DNA amplification was carried out using primers specific for the gene *invA* by using commercial PCR kits in a thermocycler (Gene amplification PCR system 9600, eppendorf, Germany). PCR reaction mixture and PCR conditions were

same as described elsewhere (Haider *et al.*, 2009). Amplified products were separated by gel electrophoreses on 1.5% agarose gel containing 5µg per ml ethidium bromide with a 100bp ladder as molecular weight marker

## **Results and Discussion**

### **Prevalence of isolated and identified organisms**

A total of 150 samples were collected during the study period. The bacterial flora isolated from the cloaca, liver and intestine of healthy and sick/dead poultry are shown in the Table 1. The cloacal samples were taken from both live and dead chickens whereas internal organ samples were taken only from dead/sick chickens. The prevalence of *Salmonella Gallinarum* was found in 28% cases, which was confirmed by different sugar fermentation and biochemical tests. This result is lower than the reports of other authors (Jordan and Pattison, 1996; Jones *et al.*, 2001)

In case of commercial layer, among 117 samples tested, 39(33.33%) were positive for *Salmonella* and in case of sonali chickens 3 (16.67%) were positive for *Salmonella* from 18 samples where as all samples (15) collected from broiler chicken were negative for *Salmonella* (Table 2).

### **Clinical signs**

The affected chickens exhibited drowsiness, weakness, loss of appetite, ruffling feathers, poor growth, labored breathing, attendant cytoseparate from healthy chickens and sometimes greenish-yellow diarrhea were observed. The adult affected chickens showed depression, anorexia, diarrhea and dehydration. There was found the drop of feed consumption

**Table 1. *Salmonella* Gallinarum isolated from different organs (n=150) of chickens**

Swabs#	Total number of samples	No. of positive cases		% of positive cases
			Total	
Cloacal	Healthy	75	9	28
	Sick/Dead	75	33	
Intestinal	Healthy	-	-	44
	Sick/Dead	75	33	
Liver	Healthy	-	-	44
	Sick/Dead	75	33	

(#Cloacal swabs were taken from both live and dead chickens where as intestine and liver swabs were taken only from dead/sick chickens)

**Table 2. Prevalence of *Salmonella* Gallinarum isolated from broiler, sonali and layer chickens (n=150)**

Type of poultry	No. of case	No. of positive cases	% of positive cases
Broiler	15	0	0
Cock (Sonali)	18	3	16.67
Layer	117	39	33.33

Lane 1: Negetive control (NC), Lane 2-6: Showing 284bp band specific for *invA* gene of *Salmonella*.

was seen in all the infeceted chickens and appeared similar with the findings of previous authors (Watkins *et al.*, 2003; Haider *et al.*, 2008; Saha *et al.*, 2012; Ogie *et al.*, 2013; Dutta *et al.*, 2015 and Unze *et al.*, 2017).

### Gross lesions

Grossly, the liver appeared swollen, congested along with bronze discoloration and in few cases liver revealed hemorrhages and focal necrosis (Fig. 1a) found similar with the previous findings of Calnek *et al.* (1997); Madhuri and Sadana (2005) and Saha *et al.* (2012). The cardiac lesions consisted of mild to moderate congestion and petechial hemorrhage in the base of the heart. In few cases, multiple white nodules with distorted shapes were observed (Fig. 1b). Ogie *et al.* (2013) and

Uneze *et al.* (2017) also reported similar findings. Splenomegaly along with multiple necrotic foci on the surface of the spleen was found. Pinpoint hemorrhages were seen in the spleen (Fig. 1c) and kidney. Lungs were highly congested with pneumonic lesions. The ova were deformed, discolored and cystic (Fig. 1d). The observation of this study was supported by the findings of Madhuri and Sadana (2005); Beyaz *et al.* (2010); Garcia *et al.* (2010); Nazir *et al.* (2012); Saha *et al.* (2012) and Uneze *et al.* (2017). Catarrhal inflammation is a frequent was also evident in some cases characterized by thick slimy mucus exudates on mucosal surfaces in the lumen of intestine (Fig. 1e). The findings were in agreement with the findings of Saha *et al.* (2012) who found 70.6% intestine hemorrhagic to catarrhal enteritis while 29.4%



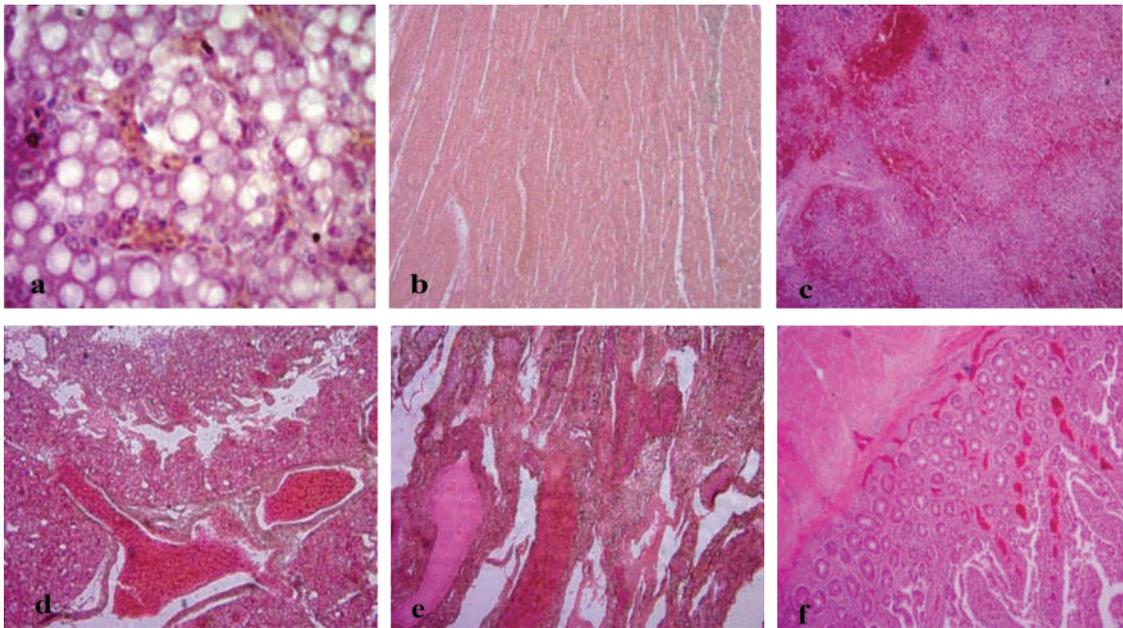
**Fig. 1.** Gross pathological changes in visceral organs of chickens caused by *Salmonella Gallinarum* (a) bronze discoloration with enlargement of liver, (b) necrotic nodule on heart, (c) splenomegaly and pin point hemorrhages were seen in the spleen, (d) catarrhal inflammation and thick slimy mucusexudates on mucosal surfaces in the lumen of intestine, (e) ova were deformed, discolored and cystic.

only hemorrhagic and congested which also supported by other authors Madhuri and Sadana (2005); Ogie *et al.* (2013) and Uneze *et al.* (2017).

### Histopathology

Microscopically, the hepatitis was characterized by leukocytic infiltration at perivascular areas along with hydropic vacuolation in hepatocytes, multiple necrotic foci noticed with Kupffer cell hyperplasia. The section of livers showed congestion, hemorrhages, focal degeneration, focal necrosis with infiltration of mononuclear cells and round cells and congestion of the central vein (Fig. 2a). Such histopathological study of liver were supported by Calnek *et al.* (1997); Hossain *et al.* (2006); Nwiyi and Omadamiro (2012) and Joshua *et al.* (2015).

Section of the hearts showed severe degeneration and fragmentation of muscle fibers (non suppurative myocarditis) with leukocytic infiltration (Fig. 2b). Fibrinous pericarditis with infiltration of heterophils, lymphocytes and macrophages was also observed in some cases. Spleen showed necrosis of lymphoid follicles leading to severe depletion of lymphocytes in white pulp along with reticuloendothelial cell hyperplasia (Fig. 2c). Lungs showed diffuse congestion and hemorrhages. Haemorrhage was also seen in the alveoli (red hepatization). Depletion of sero-fibrinous exudate was seen in lung alveoli and inter-lobular septa (Fig. 2d). The section of ovary showed different shaped ova with hemorrhages (Fig. 2e). These histopathological findings were in agreement



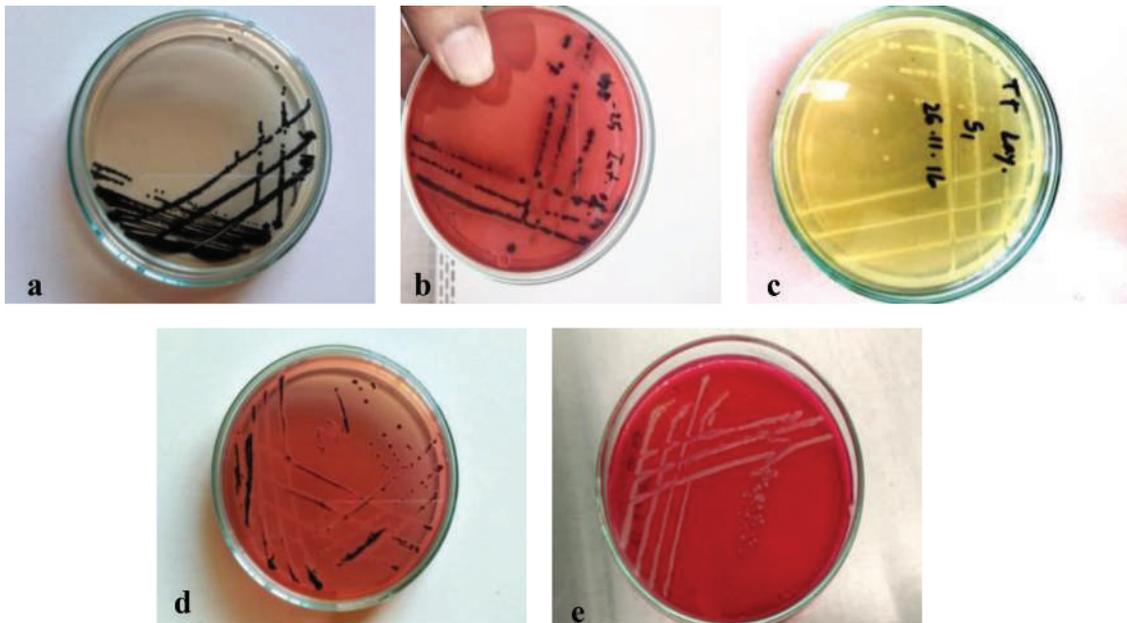
**Fig. 2.** Histopathological changes in different organs of chickens caused by *Salmonella Gallinarum*, (a) section of liver showing vacular degeneration and necrosis of hepatocytes, (H & E, X 100), (b) section of heart showing scatteredly distribution of more acidophilic muscle fibers with few heterophils and round mononuclear cells among the muscle fibers, (H & E, X 100), (c) section of spleen showing focal degeneration and necrosis of lymphocytes (H & E, X 40), (d) section of intestine showing hemorrhage and infiltration of inflammatory cells, (H & E, X 40), (e) section of ovary showing different shaped ova with hemorrhage, (H & E, X 40), (f) section of lungs showing severe congestion, infiltration of heterophils and pink color exudate in alveoli, (H & E, X 40).

with Garcia *et al.* (2010); Ogie *et al.* (2013); Joshua *et al.* (2015) and Uneze *et al.* (2017).

The section of the intestine showed desquamation of mucosal epithelium resulting in denuded villi and lumen was filled with necrotic mass, congestion, hemorrhage and infiltration of inflammatory cells (Fig. 2f). These findings were supported by the result of Saha *et al.* (2012) who reported that 47.1% infiltration of heterophils and lymphocytes in the mucosa. The predominating cells in inflamed intestinal mucosa due to FT are heterophils and lymphocytes (Hossain *et al.*, 2006; Nwiyi and Omadamiro, 2012).

### Colony morphology

On *Salmonella-Shigella* (SS) agar, all the isolates produced translucent, black, smooth, small round colonies which are positive for *Salmonella Gallinarum*. All the suspected *Salmonella* isolates produced pink color colony with black centre (Fig. 3a). Black colonies were produced on Xylose Lysine Deoxycholate (XLD) Agar medium from isolated *Salmonella* organism (Fig. 3b). White colonies were produced on Nutrient Agar (NA) medium from isolated *Salmonella* organism (Fig. 3c). Black colonies were produced on TSI medium from isolated *Salmonella*



**Fig. 3.** Colony characters of *Salmonella Gallinarum* in different agar medium, (a) production of black colonies on Salmonella-Shigella (SS) Agar medium, (b) production of black colonies on Xylose Lysine Deoxycholate (XLD) Agar medium, (c) production of white colonies on Nutrient Agar (NA) medium, (d) production of black colonies on TSI medium, (e) production of white colonies on Brilliant Green Agar (BGA) medium.

organisms (Fig. 3d). White colonies were produced on Brilliant Green Agar (BGA) medium (Fig. 3e). All these were suggestive for bacterial isolate belonging to *Salmonella* spp. (Sujatha *et al.*, 2003; Muktaruzzaman *et al.*, 2010; Hyeon *et al.*, 2012).

In microscopic examination smears stained Gram's stain revealed gram-negative, non motile, pink colored, short rod shaped bacteria, arranged in single and paired (Fig.4). These findings support the report of Ambily and Mini (2014). Hanging drop preparation of bacterial growth in nutrient broth showed the bacteria non motile belonging to similar others (Grimont *et al.*, 2000).

### Results of biochemical tests

Six of the isolates fermented glucose, maltose, lactose, mannitol and dulcitol with producing acid, which are typical characteristics for *S. Gallinarum*. There was no fermentation of lactose, sucrose and arabinose. Acid production was marked by the color change from reddish to yellow. Gas production was marked by accumulation of gas in the Durham's tube. These results were supported by Hossain (2006) who stated that among five basic sugars the *Salmonella* ferment dextrose, maltose and mannitol with production of acid and gas but no fermentation was observed in lactose and sucrose. Proux *et al.* (2002) reported that the biovar *Salmonella Pullorum* and *Salmonella Gallinarum* were differentiated by the use of sugars such as maltose, dulcitol and glucose.

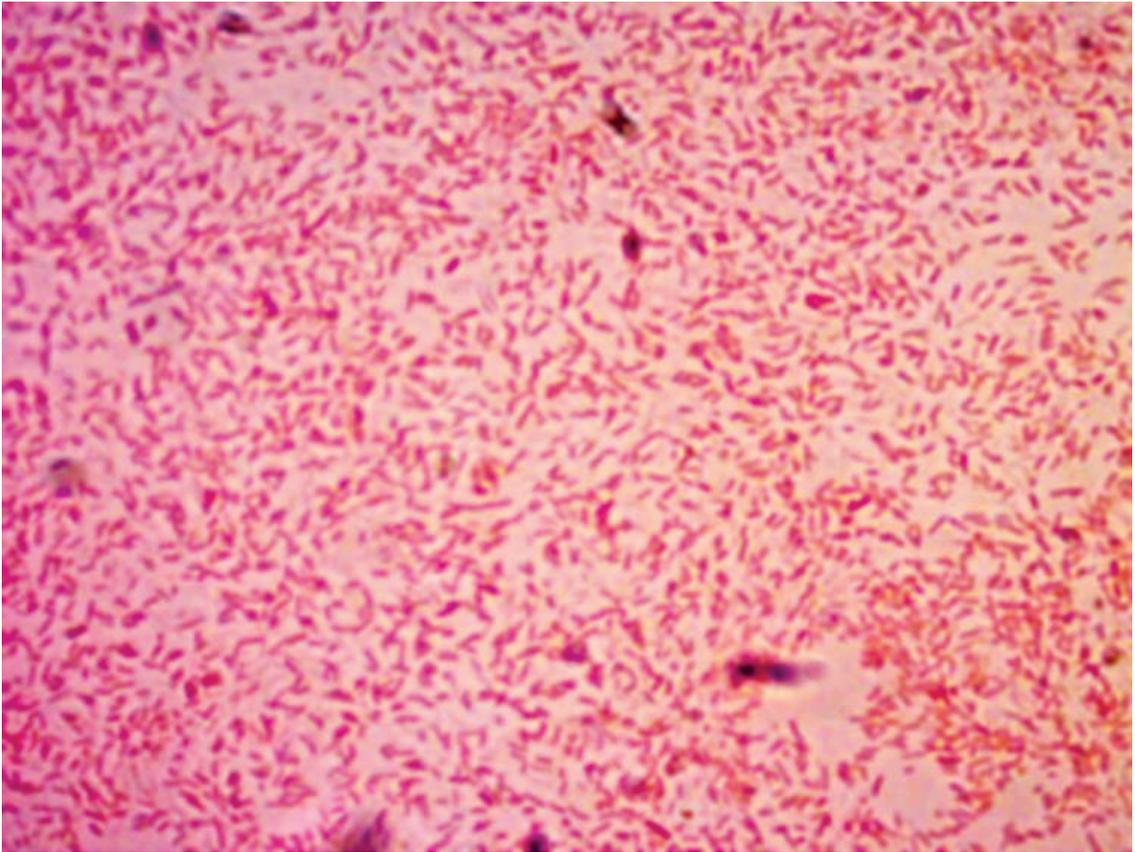


Fig. 4. Isolated *Salmonella* Gallinarum showing rod shape, short chain forming bacteria, (Modified Gram's stain, X 100).

#### Molecular detection of *S. Gallinarum* by PCR

Polymerase chain reaction with *invA* primer, 5 isolates showed positive band at 284bp i.e. the five (5) isolates were found to be *Salmonella* Gallinarum. All the *Salmonella* suspected culture subjected to PCR amplification, generated a product of approximate molecular size 284bp fragment of *invA* gene (100 specific for *Salmonella* spp.) 100bp DNA marker was used as a molecular weight marker. The band size detected in all the *Salmonella* isolates and

analyzed by agarose gel electrophoresis (Fig. 5). All conditions and results found in the PCR are supported by the findings of the several authors such as Simone *et al.* (2009); McClelland *et al.* (2001); Kisiela *et al.* (2005); Jawad and Hamadani (2011). Maximum investigators (Guo *et al.*, 1999; Ferretti *et al.*, 2001; Schneder *et al.*, 2002) try to establish a method, which can reduce the periods of *Salmonella* identification procedures from various samples and they recommended this *invA* gene for detection of *Salmonella*.

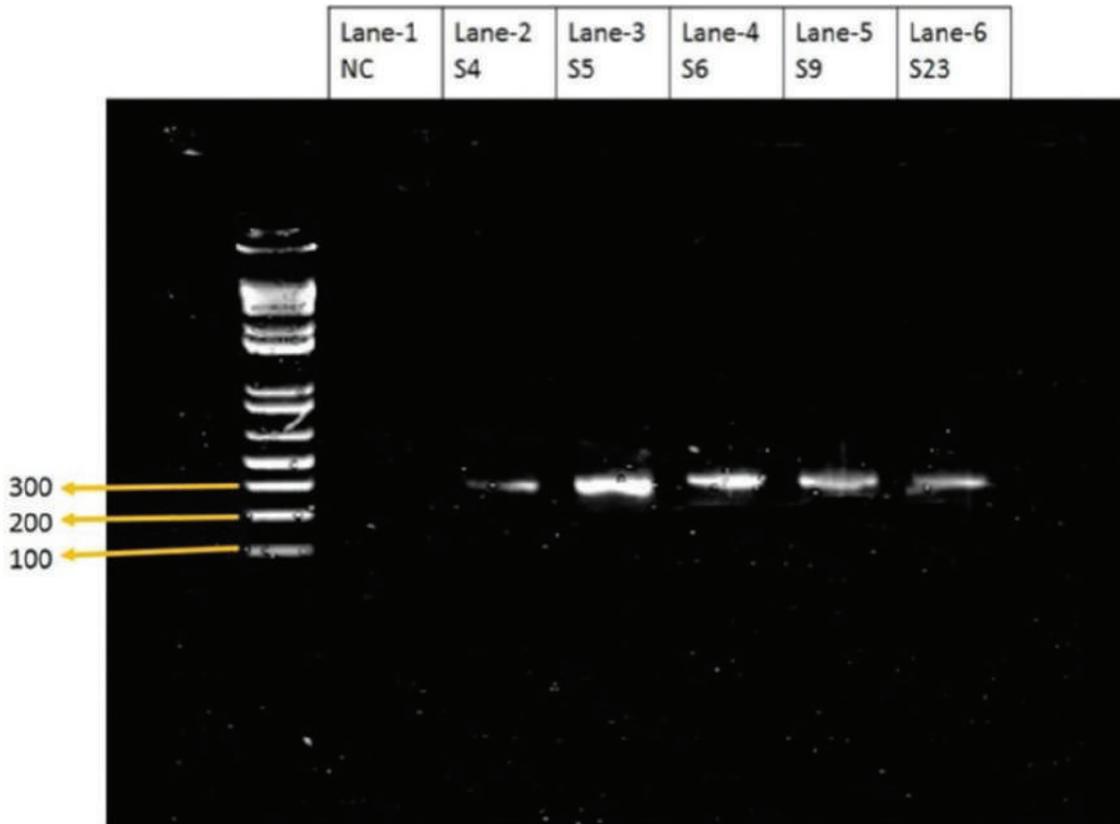


Fig. 5. Agarose gel electrophoresis for visualization of *invA* gene (284 bp) of *Salmonella*.

## Conclusions

*Salmonella Gallinarum* is a causal agent of fowl typhoid. It is a major concern of poultry industry in recent days in Bangladesh. As such, researchers, veterinarians, farmers and the government are working in tandem to search for new method of testing and characterization for *Salmonella Gallinarum*. Clinical signs, gross lesions, biochemical tests and histopathological lesions study are helpful for the detection of fowl typhoid though there have some contradictions. However, PCR based methods for identifying pathogens provide more advantageous options for this purpose than conventional testing. The

PCR protocol adapted targeting *invA* gene is specific for *Salmonella spp.* Therefore PCR methods described here might be used in combination with biochemical study to identify fowl typhoid.

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