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PATHOLOGY OF PULLORUM DISEASE AND MOLECULAR CHARACTERIZATION OF ITS PTHOGEN

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Abstract

This experiment was conducted to determine the pathology of pullorum disease of chickens and molecular identification of its pathogen. A total of 108 samples, including swabs from different organs were collected from different commercial layer farms of Gazipur district. The histopathological samples were collected in 10% formalin and the swabs were collected in tetrathionate broth. Samples were subjected to isolation and identification of the causal agent followed by gross and histopathological study of the affected visceral organs. Fifty eight out of 108 cloacal swabs (53.7%) were *Salmonella* positive. The percentage of *Salmonella* positive in liver swabs, spleen swabs, lung swabs and intestinal swabs from dead birds were 55.88%, 32.35%, 35.29% and 47.05%, respectively. On average, 52.94% livers of *Salmonella* affected birds were enlarged, congested and hemorrhagic and necrotic foci was present in 32.35% liver. Unabsorbed and coagulated yolk was found in 70.58% cases. From these 38.24% spleens were swollen and congested and 44.12 % kidneys were enlarged. At histopathology, 52.94% livers showed congestion, focal necrosis with multifocal infiltration of histiocytes in liver parenchyma. Focal necrosis and inflammatory cells were found in 70.58% spleen. Infiltration of heterophils in intestinal mucosa was found in 47.05% cases. 20.58% (7 out of 34) samples were PCR positive for *Salmonella* Pullorum organism.

Keywords: *Salmonella* pullorum, histopathology, chicken, Gazipur, focal necrosis.

Introduction

Bangladesh is an agricultural country with a large number of domestic chickens and ducks. About 80% of the total population of Bangladesh is living in the 68,000 villages, and almost each and every village households have 6-7 chickens. It is estimated that there are about 153 million chickens in Bangladesh. There are about one Lac poultry farms in Bangladesh, of which 20% rearing 1000 to 50,000 birds and remaining 80% are small in size with 100 to 1000 birds. Currently there are about 130 hatcheries including 65 breeding hatcheries with farm in Bangladesh, with two million broilers and 0.6 million layer parent stock which are producing 6 to 8 million commercial day-old-chicks per week. The commercial broiler and layer farms are supplying about 0.4 million metric ton of poultry meat and 1493.31 lakh in 2016-17 table eggs in Bangladesh (BBS, 2018). Bangladesh Economic Review (2013) reported that there are 65,902 poultry farms in the country. A total of 25000 farms were

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closed within two years from 2011-2013, due to outbreak of different diseases (Daily Star, 2013). In poultry industries work 20 lakh individuals directly and 40 lakh indirectly. Total investment in poultry sector is taka 35,000 crore and commercial farms increase to 1,20,000 in 2018 (Khaled, 2018).

Ultimately, the poultry sector of the country is neither classified as agricultural sector nor as industrial sector, as a result not getting any substantial support considering its potential contributed nutrition demand of the country. Based on the poultry industries, a number of input and output sector and a number of service providing organization are developed where 60 lakh peoples are involved directly or indirectly (Raha, 2014).

The prosperity of poultry industry in Bangladesh is interrupted by a number of constraints, of which major one is outbreak of disease causing about 30% mortality of chickens in every year. The major constraints for etiological agents are the microorganisms, parasites, management causes, environmental causes and deficiency of mineral and vitamins. The microorganisms responsible for major causes of these diseases are *Escherichia coli*, *Salmonella* spp. and fungus pathogens etc. (Islam *et al.*, 2014). There are several constraints in the development of poultry industry in Bangladesh (Das *et al.,* 2005). Infections with bacteria of the genus *Salmonella* are responsible for major problem of poultry farming in Bangladesh (Kamaruddin and Giasuddin, 2003). Avian salmonellosis, caused by a variety of *Salmonella* species, is one of the most important bacterial diseases in poultry causing heavy losses through mortality and reduced production (Haider *et al.,* 2014). Among various diseases of poultry, caused by *Salmonella* Pullorum caused is one of the major diseases which have significant zoonotic importance in those people who eat massively contaminated poultry meat and eggs (Shivaprasad and Barrow, 2013).

Salmonella infection is one of the most important global poultry diseases in avian species because of its huge economic impact, worldwide distribution and difficulty posed in the control of the disease (Kabir, 2010). Among this *S.* Pullorum is the main cause of considerable economic importance in the poultry industry, particularly in developing countries with a poultry industry. The pathogen not only can cause high mortality rates among young chicks but also persists for a long period in the spleen and the reproductive tract, leading to the infection of ovaries or progeny (Li *et al*., 2013). A great economic loss, due to this disease is that it causes high mortality rates which can reach up to 100%, decrease in production (eggs and chicks), condemnation of affected carcass and cost of medication both in humans and animals. Direct health costs such as consulting a physician hospitalization, laboratory testing, medication and as well as the more labour costs of in relation to handling the case of salmonellosis are estimated as part of a multidisciplinary task (Netsanet *et al.*, 2012). Eradication of the carrier parent flocks and growing chickens, and their placement by new chickens also cause significant economic loss (Shivaprasad and Barrow, 2013). Additionally, according to WTO, exportable eggs and meat of chickens must be free from *Salmonella*. That cause great effect on currency of one country getting from the poultry sector through exporting (Hossain, 2011). Pullorum disease is a serious

economic problem to livestock in countries where measures of control are not efficient or in those where the climatic conditions are favour for spread of these microorganisms (Barrow and Neto, 2011).

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 authenticated as a specific PCR target with important diagnostic applications for the genus *Salmonella* because it posses the unique sequence specific for the genus (Rahn *et al.,* 1992 and Malorny *et al.,* 2003). The pathogenicity island 1 (SPI-1) is the location where the *invA* is located on which is highly specific in most *Salmonella* serotypes and has been used for detection of *Salmonella* as an important target in PCR (Li *et al.,* 2012 and Karmi, 2013).

Only a limited performed investigations have been performed on natural case of *Salmonella* infections in Bangladesh using the methods of necropsy and histopathology, and isolation of bacteria by culture in media, staining and sugar fermentation tests, and experimental pathogenesis, pathology and vertical transmission in chickens (Haider *et al*., 2008; Hossain *et al*., 2006; Islam *et al*., 2006; Haider *et al*., 2004).

Seventy percentage chicken and eggs of total production in Bangladesh are produced in Gazipur district. But no investigation has been performed for isolation, molecular characterization of *Salmonella* available in Gazipur, Bangladesh. For the control of *Salmonella* infection in poultry to save the poultry industry, histopathogical, biochemical and gene level study need to be performed from the isolates those are endemic at Gazipur district of Bangladesh (Saha *et al.,* 2012). Considering all the aspects, in the present study we have tried to observe the prevalence of *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 characterization of the *Salmonella* by PCR targeting *invA* gene.

Materials and Methods

Ethical approval

According to specification of International Ethics Guidelines authors proceeded to ensure a correct, careful and safe handling of chickens.

Study area

Dead and diseased birds which were suspected to be infected with *Salmonella* infection were collected from different commercial layer farms of different upazilla (Fig. 1) of Gazipur district, Bangladesh.

Animals and experimental design

The research was conducted in facilities facilitated by Department of Pathobiology of Faculty of Veterinary Medicine and Animal Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh. A total 108 samples were collected randomly from ten different layer commercial poultry farms (Chickens) viz. Diamond egg Ltd, Protein House Ltd., Diamond Chicks Ltd, Nourish Poultry farms and Paragon Poultry farms etc. covering the whole district. Swabs were collected from cloaca and from liver, spleen, lungs, heart,

Fig. 1. Experimental area.

ovaries after dissection of the respective birds. Aseptic cotton swabs were used and the swab samples were collected in test tubes containing 10 ml tetrathionate broth (TTB) according to methods as described previously (Haider *et al*., 2008). After collection, first all the bacteriological samples were incubated for 24 hours in TTB at 37^0C. Then, all the samples were primarily cultured in Nutrient agar (HiMedia) and then cultured again in the *Salmonella-Shigella* (SS) agar, Triple sugar iron (TSI) agar, Brilliant green agar (BGA), Eosine methylene blue (EMB) agar for obtaining desired single colony (Haider *et al*., 2008). Then the presumptive colonies of suspected bacteria in various media were characterized microscopically using Gram's stain. After that, sugar fermentation tests were done. Eight sugars such as glucose, sucrose, lactose, mannitol, dulcitol, arabinose, xylose and maltose were used for sugar fermentation test (Haider *et al*., 2008). For the separation of motile and non-motile bacteria, motility tests were performed according to the method followed by Haider *et al*. (2008).

DNA extraction

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).

Polymerase chain reaction (PCR)

Extracted DNA amplification was carried out using primers for the gene invA by using commercial PCR kits in Gene amplification PCR system 9600 Thermocycler (eppendorf, Germany) (Shanmugasamy *et al.,* 2011).

Agarose gel electrophoreses

Separation of amplified products were done by electrophoreses on 1.5% agarose gel containing 58 µg per ml ethedium bromide with a 100 bp ladder as molecular weight marker (Haider *et al*., 2008).

Processing of tissues

Collected tissue samples of intestine, liver, lungs, spleen, heart and ovary were fixed in 10% neutral buffered formalin and further processed for histopathological examination (Haider, 2013).

Photomicrography

Photomicrography was taken using photomicrographic camera (ZEISS AxioCam ERc5s) facilities facilitated by Department of Gynecology, Obstetrics and Reproductive Health of BSMRAU.

Results and Discussion

Prevalence of isolated and identified *Salmonella*

A total of 58 samples were found to be *Salmonella* positive among the total 108 samples based on colony characters on different agar media, morphological characteristics and biochemical test results. So, the prevalence rate of salmonellosis is 53.7%. The present prevalence of salmonellosis are agreement (Islam *et al.,* 2016) but slightly higher than the findings of other authors (Ahmed *et al.,* 2008; Rahman *et al.,* 2011).

Colony characters

Salmonella organism were formed round, raised, transparent single colonies with black centers on SS agar (Fig. 2a). In the present study the colony characters of *Salmonella,* the production of hydrogen sulfide gas with black colonies on SS agar were similar to the results of other authors (Hossain *et al.,* (2006); Rahman *et al.,* (2008); Lujain *et al.,* 2016). On XLD agar, *Salmonella* produced black colonies (Fig. 2b). In EMB, agar *Salmonella* was formed the pink colonies (Fig. 2c). *Salmonella* produced red to pink white colonies surrounded by brilliant red zones on BGA agar (Fig. 2d). Black colonies were showed when these were grown on TSI medium (Fig. 2e). All these findings are corresponding to Saha *et al.,* (2012).

Morphology

The morphology of the organisms were gram negative, rod shaped, pink color bacilli with Gram's Stain (Fig. 3) which is similar to the findings of other authors (Haider *et al.,* 2004; Rahman *et al.,* 2008; Lujain *et al.,* 2016).

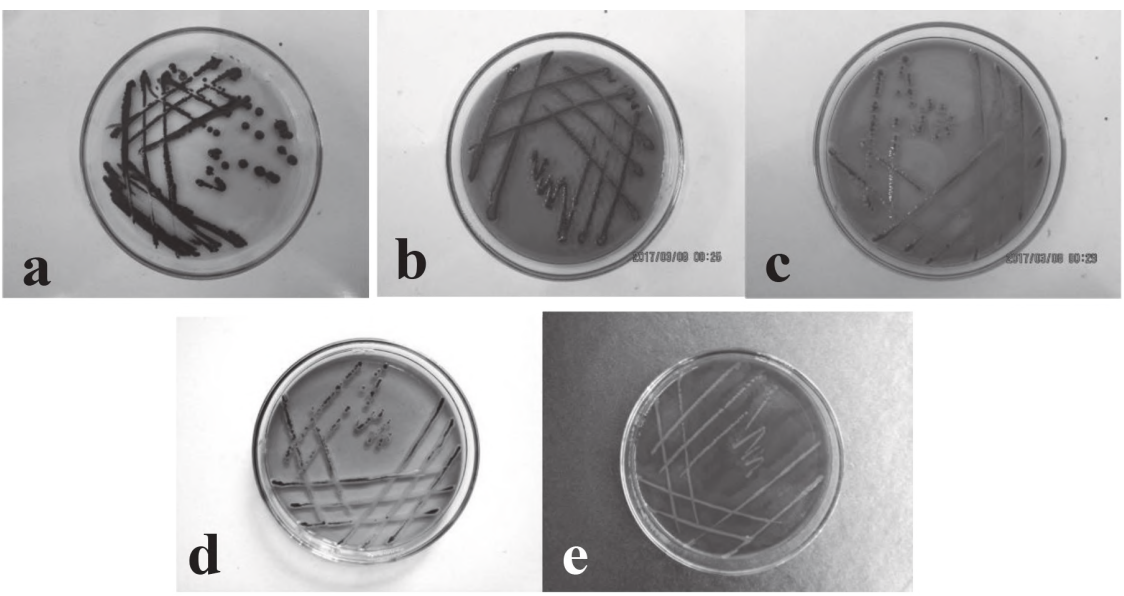

Fig. 2. Colony morphology of *Salmonella* on different agar media. (a) Transparent single colonies with black centers on SS agar, (b) *Salmonella* produced black colonies on XLD agar, (c) *Salmonella* produced pink colonies on EMB agar, (d) *Salmonella* produced black colonies on TSI agar and (e) *Salmonella* produced small, opaque, pinkish white colonies on BGA.

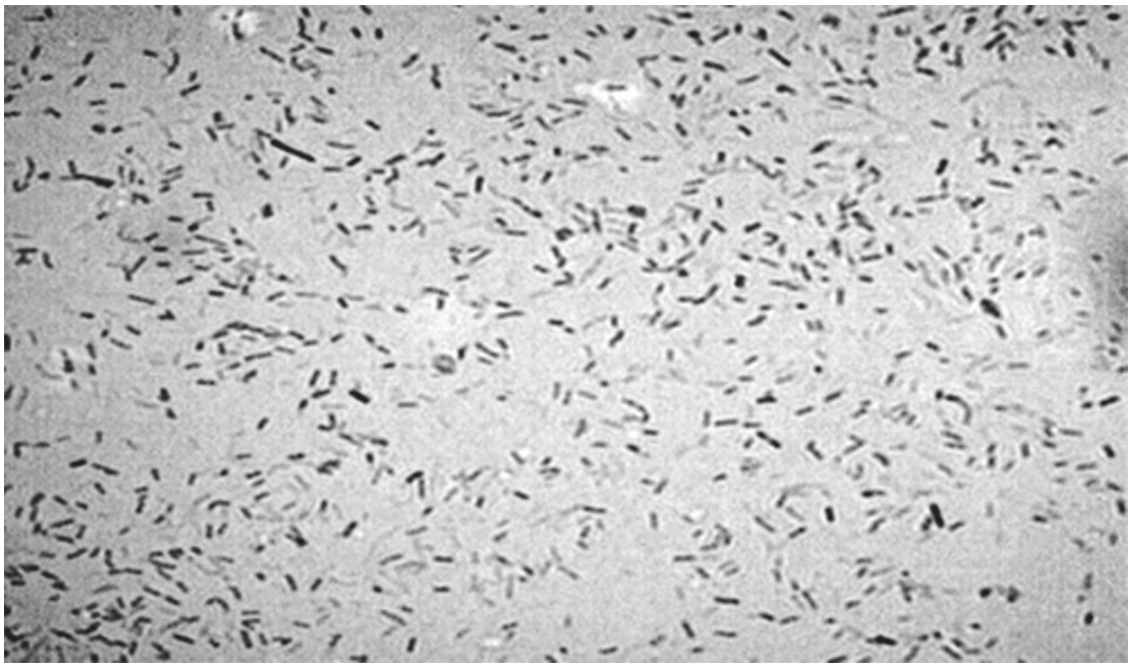

Fig. 3. Isolated *Salmonella* showing negatively stained rod-shaped bacteria (Gram Stain X1000).

Motility

The isolated organisms were non-motile when examined under microscope with Hanging drop slide preparation which is supported by the result of Haider *et al. (*2004).

Biochemical Test

The organisms fermented dextrose, maltose, xylose, mannitol, arabinose and dulcitol but did not ferment the sucrose and dulcitol which is corresponding to the results of other authors (Haider *et al.,* 2004; Saif *et al.,* 2008; Ahmed *et al.,* 2008).

Results of molecular detection of *Salmonella* spp.

DNA extracted from all of the isolates of *Salmonella* spp. were used in PCR assay. All the isolates of *Salmonella* which were presumptively identified on the basis of cultural, Gram's staining and biochemical tests were confirmed by Polymerase chain reaction using genus specific primers. A total of 7 (20.59%) isolates were confirmed as *Salmonella* (796 bp) by amplifying genus specific *Inv*-A primers (Fig. 4). The similar result also found by other authors (Cohen *et al*., 1993; Carli *et al.,* 2001; Fratamico, 2003; Paião *et al.*, 2013).

Gross lesions

Grossly, the livers were congested and focal necrosis was found (Fig. 5a) and petechial hemorrhages were found in the spleen (Figure 5b) which are similar to the findings of other authors (Majid *et al.,* 2000; Hossain *et al.,* 2006; Rahman *et al.,* 2011; Saha *et al.,* 2012; Nazir *et al.,* 2012; Palani *et al.,* 2014). The ova were hemorrhagic, deformed and cystic (Fig.

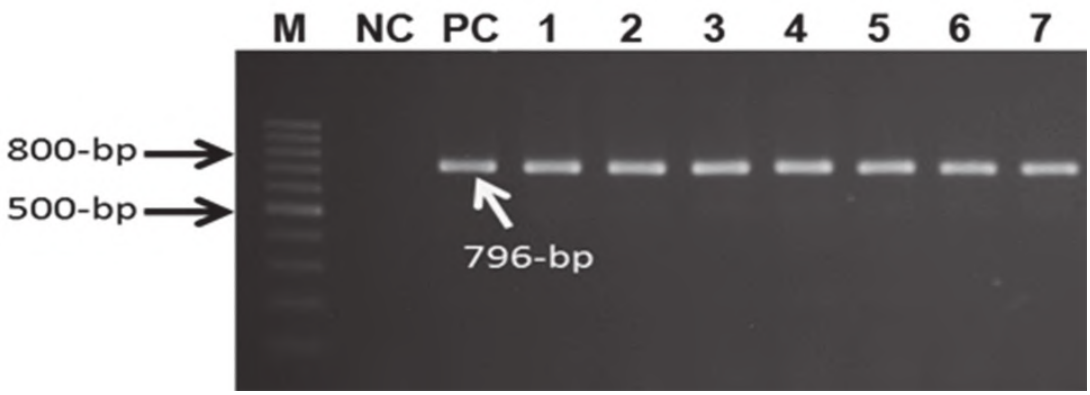

Fig. 4. Amplification of *Inv-A* gene for *Salmonella* spp. Lane M: 100-bp DNA ladder, NC: Negative control, PC: Positive control and Lane1-7: Isolated sample of *Salmonella* spp. This figure represents the first PCR amplification of the culture positive 7 *Salmonella* spp. using genus specific primer *Inv- A* F and Inv-A R showing amplification size 796-bp.

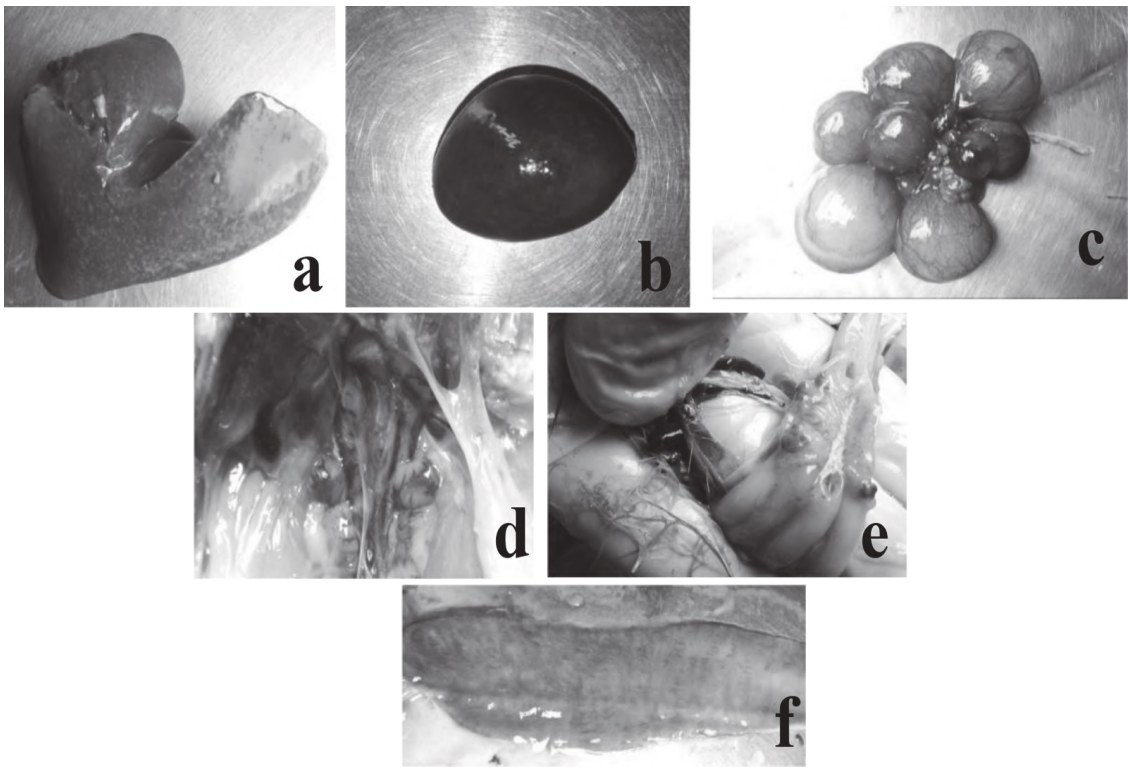

Fig. 5. Gross lesions of different organs of chickens caused by *Salmonella* (a) Haemorrhagic and congested liver with white necrotic foci, (b) Swollen and congested spleen in *Salmonella* infected chickens, (c) Hemorrhagic, deformed and cystic ovary in *Salmonella* infected chickens, (d) Petechial hemorrhage in kidneys in *Salmonella* infected chickens, (e) Pin point hemorrhage was present in caecal tonsils in *Salmonella* infected chickens, and (f) Haemorrhagic intestine in *Salmonella* infected chickens.

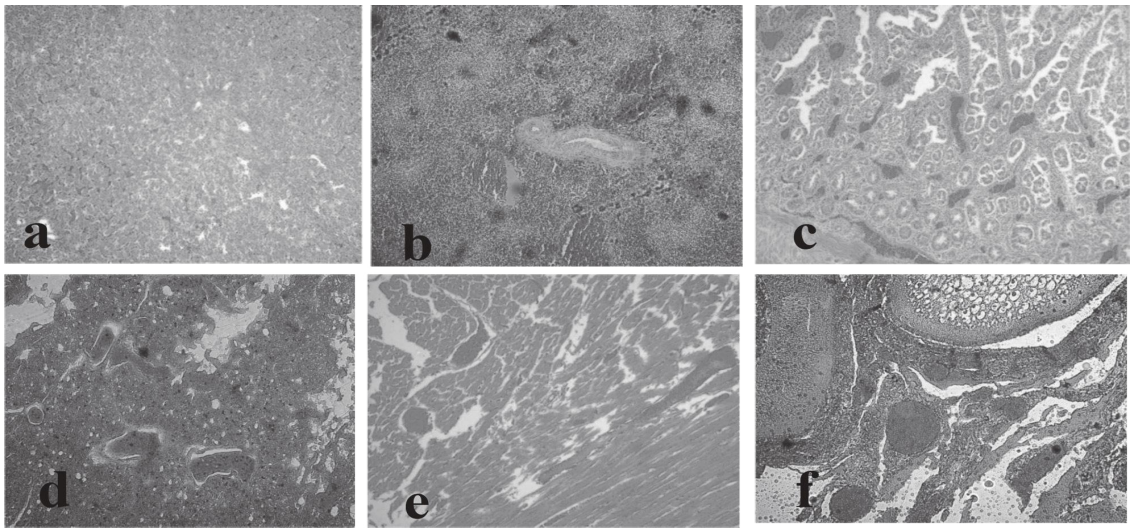

Fig. 6. Microscopic lesions of different organs of chickens caused by *Salmonella* (a) Section of liver showed vascular congestion, multifocal degeneration and necrosis of hepatocytes and infiltration of heterophils (H & E X100), (b) Section of spleen showed severe congestion and focal necrosis (H & E X100), (c) Section of intestine showed severe congestion and infiltration of inflammatory cells, (H & E X100), (d) Congestion in blood vessels and sero-fibrinous exudation in lungs (H & E X100), (e) Section of heart showing necrosis of the myofibers and few infiltrations of heterophils (H & E X100), and (f) Section of ovary shows congestion and infiltration of inflammatory cells (H & E X100).

5c). At base of heart and kidneys, petechial hemorrhages were found (Fig. 5d). The pin point hemorrhage was present in caecal tonsils (Fig. 5e). These findings are also found by other authors (Majid *et al.,* 2000; Ahmed *et al.,* 2008; Nazir *et al.,* 2012). Profuse hemorrhage was present in intestine (Fig. 5f) which is similar to other authors (Majid *et al.,* 2000; Nazir *et al.,* 2012).

Microscopic lesions

The prepared histopathological slide of liver section showed vascular congestion, multifocal degeneration and necrosis of hepatocytes and infiltration of heterophils (Fig. 6a). Section of spleen showed severe congestion and focal necrosis (Fig. 6b). The lesions of small intestine included severe congestion and infiltration of inflammatory cells (Fig. 6c). Pulmonary lesions described that there were congestion in blood vessels and sero-fibrinous exudation in lungs (Fig. 6d). The section of heart showed necrosis of the myofibers and few infiltrations of heterophils (Fig. 6e). The ovary showed congestion and infiltration of inflammatory cells (Fig. 6f). All these histopathogical lesions are also described by other authors (Majid *et al.,* 2000; Haider *et al.,* 2004; Ahmed *et al.,* 2008; Holt *et al*., 2010; Rahman *et al.,* 2011; Saha *et al.,* 2012).

Conclusion

Salmonellosis is one of the main causes of morbidity, mortality and high economic losses in poultry industry in Bangladesh. The prevalence of the salmonellosis in this study was found 53.7%. Highest cultural prevalence was in swabs collected from

cloaca. In gross pathology, it had been found hemorrhage and congestion in intestines, liver, spleen and ovaries. Focal necrosis was found in liver and spleen. Ovaries showed different shaped ova where the ova were hemorrhagic, deformed and cystic. Pin point hemorrhage was present in caecal tonsils. In histopathological investigations, it had been found prominent lesions in liver, spleen, intestine, heart, lungs and ovary. The findings of this study will help the veterinarians and researcher on pullorum disease. Further study on molecular characterization of *Salmonella* Pullorum should be conducted to prevent the infection through vaccine production or by others molecular way like gene deletion.

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