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Assessment of foodborne transmission of *Salmonella enteritidis* in hens and eggs in Bangladesh

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Abstract

Objectives: *Salmonella* is considered one of the leading causes of foodborne illnesses worldwide. Information about the transmission of pathogens to poultry and poultry products is necessary to implement control measures for reducing both human exposure and economic loss. The aim of this study was to analyze and evaluate the transmission characteristics of *Salmonella enteritidis* to laying-type hen flocks and their laid eggs.

Materials and methods: For this purpose, 15 pairs of laying hens were used in which each pair consisted of one inoculated and one contact exposed hen. The eggs and cloacal swabs from these hens were subsequently analyzed.

Results: Of the 15 in-contact hens tested, 60% were found to be positive for *S. enteritidis* within 61 days postinoculation, of which 26.7% transmission occurred within the first 31 days postinoculation. Among the collected laid eggs tested, *S. enteritidis* was detected on 58% eggshells and 5.33% eggs internal contents. We also observed a 33.33% reduction in egg production from *S. enteritidis*-infected hens. In a cross-contamination study, we demonstrated that an experimentally inoculated container can act as a potential source of *Salmonella* spp. infection.

Conclusions: Our results will help establish effective monitoring programs to reduce the transmission of *Salmonella* spp. in poultry and poultry products.

KEYWORDS
Bangladesh, foodborne salmonellosis, poultry, *Salmonella enteritidis*, transmission, zoonoses

1 | INTRODUCTION

Salmonellosis, a foodborne infection caused by *Salmonella* spp., constitutes a major concern in both humans and animals worldwide (Bhat & Macaden, 1983). Consumption of *Salmonella* spp. containing foods may cause mild to moderate gastrointestinal illness, which can be life threatening in severe cases (Voetsch et al., 2004). Globally, non-typhoidal *Salmonella* (NTS) is responsible for 80.3 million cases of foodborne gastroenteritis in humans, causing 155,000 deaths each year (Majowicz et al., 2010). Among 2463 serotypes of *Salmonella* spp., *Salmonella typhimurium* and *Salmonella enteritidis* are most frequently associated with foodborne salmonellosis (Galiş et al., 2013; Popoff et al., 2000). In the European Union, a study demonstrated that the annual incidence of *Salmonella*-associated infections was significantly

associated with the occurrence of *S. enteritidis* in laying hens and varied between 16 and 11,800 per 100,000 people in EU member states (Havelaar et al., 2013).

Although various foods have been implicated in human salmonellosis, eggs and egg products are the most acknowledged vehicles in outbreaks of *Salmonella* (Braden, 2006). Bacterial pathogens can contaminate eggs by following both horizontal and vertical routes of transmission (Chousalkar et al., 2013). Horizontal transmission occurs during the transition of an egg through the contaminated cloaca and includes all kinds of non-vertical transmission from the environment, such as infected hens, contaminated feed/faeces/water, personnel and equipment, and aerosols, and subsequent penetration of microorganisms, whereas in vertical or transovarian transfer, internal contents of eggs can be infected with microorganisms at the time of their formation in hen's reproductive organs (De Reu et al., 2006). After ingestion, *S. enteritidis* begins colonizing the intestinal tracts of laying hens to initiate infections that rapidly disseminate to other tissues (He et al., 2010). The number of *S. enteritidis* in the internal organs of orally inoculated hens decreased significantly during the first week, but prolonged horizontal transmission was observed in individual hens with persistent infections (Gast et al., 2009, 2007). Eggshells can be colonized by *Salmonella* spp. when it comes in contact with contaminated feed, water, hatcheries, processing equipment, poultry litter, and other environmental sources (Sivaramalingam et al., 2013). Bacterial strain, pathogen load on eggshells, temperature fluctuations, and moisture are the major contributing factors for the trans-shell transmission of *Salmonella* spp. (Messens et al., 2006). For example, the rate of penetration or internal contamination for free-range, conventional battery caged, and brown, organic, and omega-3-enriched eggs was found to be 6%, 16%, and 30–34%, respectively, after experimental inoculation of commercially available eggs with 2.71 log CFU of *S. enteritidis* and storage at 20°C for 14 days (Messens et al., 2007). In addition, numerous flock management activities and facilities influence the survival and transmission of *Salmonella* spp. in commercial poultry and their growing environment (Gast et al., 2019).

In Bangladesh, the poultry industry is one of the most promising and rapidly growing industries. It acts as a tool for poverty eradication and economic development. Approximately 10 million people are directly or indirectly involved in poultry farming in Bangladesh, and the total investment is more than Tk 15 billion, which is 10 times higher than in the 1990s (Islam et al., 2014). As the prevalence of salmonellosis in commercial poultry production is increasing, it has become a prime concern that impedes the development of the poultry sector (Das et al., 2004; Mahmud et al., 2011). However, very few studies have been reported in Bangladesh concerning the transmission of *Salmonella* in poultry and poultry products. Therefore, transmission of *Salmonella* spp. in poultry farms needs to be assessed properly to take efficient control measures. The objectives of the present study were (i) to evaluate the horizontal and vertical transfers of *S. enteritidis* in laying hens and eggs, (ii) to study cross-contamination-associated dissemination of the pathogen, and (iii) to address the effect of *Salmonella* spp. infection in egg production characteristics.

2 | MATERIALS AND METHODS

2.1 | Bacterial culture and inoculum preparation

Salmonella enteritidis was taken from laboratory bacterial stocks, which were previously identified by biochemical tests and 16S rRNA sequencing. PCR was carried out using *sdfI* primers (*sdfI*-F, 5′-TGTGTTTTATCTGATGCAAGAGG-3′ and *sdfI*-R 5′- CGTTCTTCTGGTACTTACGATGAC -3′) for the specific detection of *S. enteritidis* (Agron et al., 2001). The strain was revived and grown on XLD agar plates; from there, a single colony was inoculated in buffered peptone water (Oxoid, UK) and incubated at 37°C. The cell density was periodically measured at 600 nm to achieve a final cell concentration for each oral dose of 1.0×10^9 CFU/ml. A subsequent plate count was performed to confirm the desired cell number.

2.2 | Laying hens

Two groups of laying hens of Lohman brown classic and white Leghorn classic were taken from a commercial poultry farm. Each group contained 6-month-old 15 hens. Another group of eight laying hens (four Lohman brown classic and four white Leghorn classic) of the same age was kept in a decontaminated cage as a control. The hens were examined for the presence of *Salmonella*-specific serum antibody to determine any previous infection. Cloacal samples were collected on three consecutive days and tested bacteriologically after a short adaptation period in the new environment. Finally, another serological test was performed before inoculation of hens to ensure the absence of *Salmonella* spp. in the experimental settings. All experiments involving animals were reviewed and approved (Ref. no. 130/Biol. Scs./2021-2022) by the Ethical Review Committee of the Faculty of Biological Sciences, 'University of Dhaka'.

2.3 | Experimental design

Model 1: *Salmonella enteritidis* transmission from infected hens to in-contact hens was observed in this model. The experiment was performed following the procedure described by Thomas et al. (2009) with some modifications. Two tested groups of hens were tagged individually and kept pairwise. Fifteen pairs of hens were kept in 15 different cages. The sides and floor of the cages were secured by a plastic-coated carton, and to make a better environment, the floor was covered with decontaminated feathers and poultry feed. Each pair contained one white and one brown hen that laid white and brown eggs, respectively. Hens used as a control group were reared in another room under the same conditions.

On the first day of this experiment, one hen, either white or brown, was randomly selected, separated from the cage and inoculated orally with 1 ml of *S. enteritidis* inoculum at a dose of 1.0×10^9 CFU (Thomas et al., 2009). After 1 day, all inoculated hens were put into their

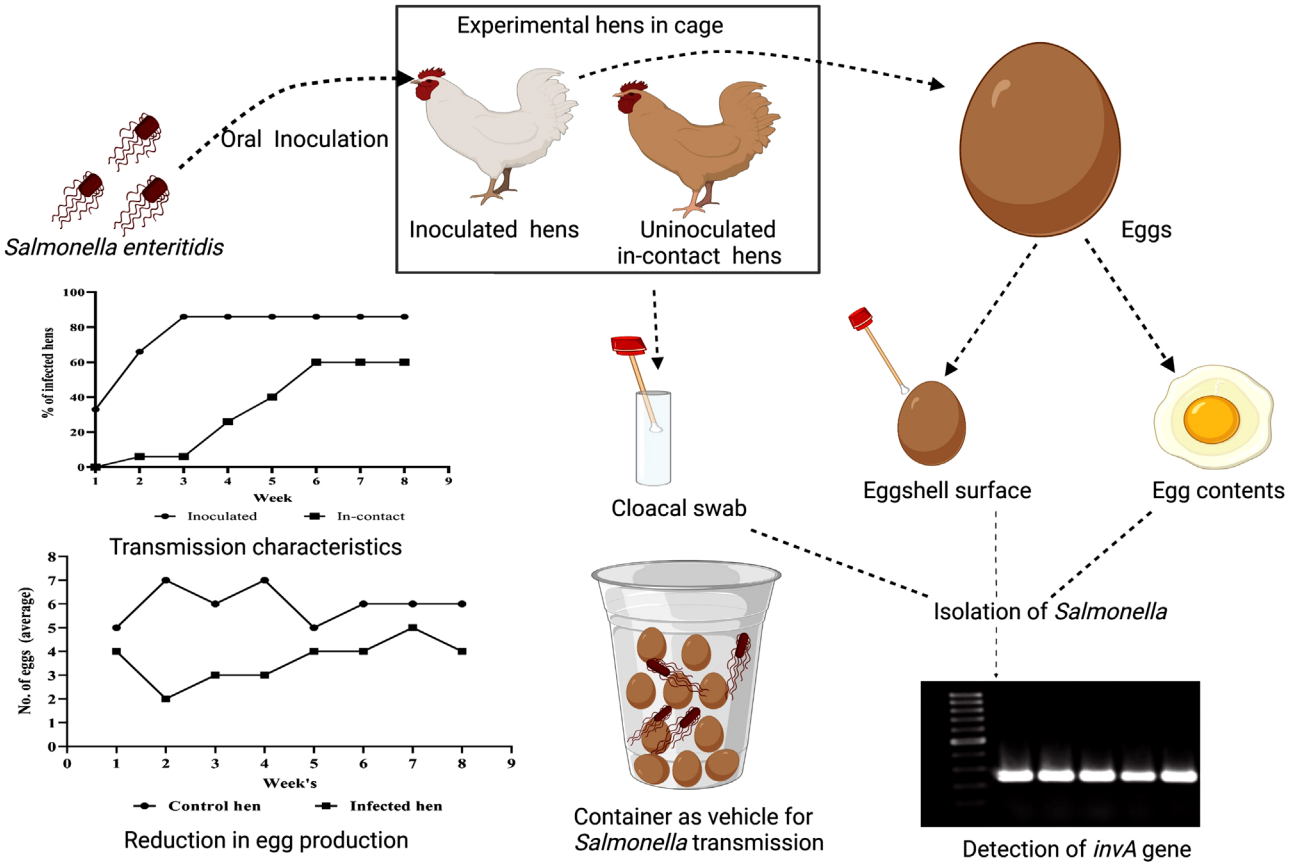

FIGURE 1 Graphical representation of the whole work

original cages again, and this day was treated as day 0. Each cage carried one inoculated hen and one contact-exposed non-inoculated hen (in contact). The hens were kept in the same cage for 61 days. The control group of hens was not inoculated. From all groups, cloacal swabs and eggs were collected as soon as the first egg was laid after inoculation. All hens were provided with sterilized and antibiotic-free drinking water and ad libitum rice grains. Cloacal swabs were collected from both inoculated and in-contact hens at regular intervals to check for the presence of *S. enteritidis* (Figure 1). Swabs were taken using a new sterile cotton plug and placed into tubes containing 10 ml sterile normal saline for bacteriological examination.

Model 2: A total of 300 eggs were collected from inoculated and in-contact hens, of which 150 eggs were tested for the presence of *S. enteritidis* on the shell surface (Figure 1). To recover *S. enteritidis* from the eggshell surface, collected eggs were kept in separate sterile plastic bags containing 10 ml of sterile phosphate buffer saline, rinsed by shaking for 1 min and cultured for the isolation of bacteria.

Model 3: Another 150 eggs were examined to detect *S. enteritidis* in internal egg contents (Figure 1). Eggshell surfaces were washed with 70% ethanol to disinfect and break them using a sterile knife. Then, egg internal contents were dispersed by vigorous mixing, and pre-enrichment of bacteria was performed in buffered peptone water. At least five eggs from the same hen were individually processed.

Model 4: Another set of experimentally infected eggs was used to assess the container contamination and further establish it as a source of contamination. A total of 140 eggs were washed with 70% ethanol to kill bacteria on the outer shell to be used in the cross-contamination experiment. A bacterial inoculum of *S. enteritidis* was prepared at a final cell concentration of 10^5 CFU/ml (Chousalkar et al., 2013). Eggs were dipped in bacterial culture for 1 min and kept in a biosafety cabinet to allow them to dry. All inoculated eggs were placed in the plastic container used for the egg transition. After 1 day, swab samples were taken from the container with a sterile cotton plug, processed, and tested for the presence of *S. enteritidis*. As the container showed a positive result, it was used again to keep another set of germ-free eggs. After 2 days, the egg shell was tested to detect *S. enteritidis* using the same procedure. The same experiment was repeated for a third batch of eggs. A total of 45, 50, and 45 germ-free eggs were tested in batches 1, 2, and 3, respectively.

2.4 | Isolation and biochemical characterization of *Salmonella*

Samples from Models 1, 2, and 3 were pre-enriched in buffered peptone water, transferred to selenite cysteine broth for selective

enrichment and plated into Xylose Lysine Deoxycholate (XLD) and *Salmonella Shigella* (SS) (Oxoid, UK) agar media to detect the presence of *Salmonella*. On the other hand, samples from Model 4 were serially diluted, spread on XLD and SS agar, and incubated overnight at 37°C to enumerate bacteria. Negative cultures were incubated further for another 24 h. Presumptive identification of *S. enteritidis* isolates was based on biochemical tests such as the triple sugar iron test, Simmons citrate test, urease test, indole test, methyl-red and Voges-Proskauer test, and motility test.

2.5 | Chromosomal DNA extraction and PCR detection

Chromosomal DNA was isolated by the boiling extraction method (De Medici et al., 2003). A freshly grown isolated colony was inoculated into Luria Bertani broth and incubated overnight at 37°C. One millilitre of culture was taken and centrifuged for 5 min at 10,000 rpm. The supernatant was removed, followed by the addition of 200 µl PCR grade water. After boiling for 10 min at 100°C, the cultures were immediately placed on ice for 10 min and centrifuged at 10,000 rpm. Then, 100 µl supernatant was collected and stored at −20°C to be used as a template in PCR.

Confirmation of *Salmonella* spp. was performed by molecular detection of the *Salmonella*-specific gene *invA*. The primer set used was F 139 and R 141 with the nucleotide sequences 5ť GTG AAA TTA TCG CCA CGT TCG GGC AA 3ť and 5ť TCATCG CAC CGT CAAAGG AAC C-3ť, respectively (Nagappa et al., 2007). The thermocycling conditions were 95°C for 1 min, followed by 35 cycles at 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 4 min, which was carried out in a Master Cycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany).

3 | RESULTS

3.1 | Model 1

With a cloacal swab test, 13 out of 15 inoculated hens were found to be positive for *S. enteritidis* between days 1 and 15 (Figure 2). Continuous shedding was observed in both inoculated and in-contact hens during 61 days of the experimental period.

Among 15 in-contact hens, nine hens showed *Salmonella* growth in culture media (Figure 2). No positive result was observed up to day 10. On day 11, only one hen showed culture positivity in the cloacal swab test. A total of four hens were observed as *Salmonella* infected within the first 31 days postinoculation. Approximately 60% transmission of *S. enteritidis* was found from infected hens to exposed in-contact hens within the experimental period, of which 26.7% occurred within the first month (31 days) of postinoculation, and the rest of the transmission occurred within the last 31 days.

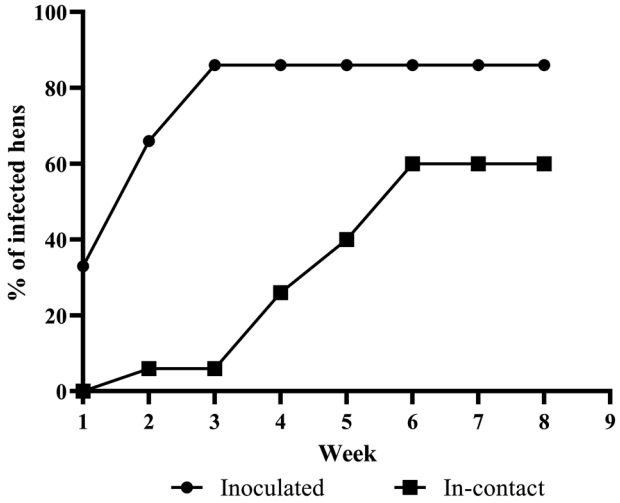

FIGURE 2 The percentage of *Salmonella enteritidis*-infected hens among the inoculated and in-contact groups during the experimental period

3.2 | Model 2

Out of 150 eggs used from the tested group (inoculated hen egg: in-contact hen egg = 1:1), 87 (58%) eggs were found to be positive for the presence of *Salmonella* on eggshell (Table 1). Eggs collected from the control group of hens were negative for *S. enteritidis* during the whole experiment.

Among 87 *Salmonella*-positive eggshells, 63 and 24 eggs corresponded to infected hens and in-contact hens, respectively. Therefore, the percentage of infection was higher in inoculated hens 63/75 (84%) than in-contact hens 24/75 (32%).

3.3 | Model 3

In the vertical transmission experiment, eight out of another 150 eggs (5.33%) were culture positive for the presence of *S. enteritidis* in egg contents. The numbers of eggs from infected hens and in-contact hens were six and two, respectively.

3.4 | Model 4

Among 140 tested egg samples that were kept in contaminated containers, only seven eggs yielded positive results after 2 days (Table 2). Therefore, a total of 5% (number of *Salmonella*-infected eggs/total number of tested eggs) transfer was conducted through a contaminated container.

3.5 | Egg production observation

During the experimental period (8 weeks), all hens in the control group laid a higher number of eggs than the infected hens. The control group

TABLE 1 Summary of the results of the transmission of *Salmonella enteritidis* in hens and eggs

Type of transmission	Sample type	Experimental group		Total rate of transmission Total SE positive in inoculated and in contact/total number of samples tested (%)
		Inoculated (15 hens)	In-contact hen (15 hens)	
		SE positive samples/No. of samples tested (%)		
Horizontal (hen to hen)	Cloacal swab	13/15	9/15 (60%)	9/15 (60%)*
Horizontal (hen to egg)	Eggshell surface swab	63/75 (84%)	24/75 (32%)	87/150 (58%)
Vertical	Egg internal contents	6/75 (8%)	2/75 (2.7%)	8/150 (5.33%)

*As inoculated hens were supposed to transmit *Salmonella enteritidis* to contact exposed hens, only cloacal swabs from in-contact hens were considered to calculate hen-to-hen transmission.
Abbreviation: SE, *Salmonella enteritidis*.

TABLE 2 The number of contaminated eggs and count of pathogens when contaminated eggs and germ-free eggs were kept inside a container

Batch	Container contamination	Contamination of germ-free egg				
	Interpretation	Average count (CFU/ml)	Amount of egg samples	Interpretation	Positive number of eggs	Average count (CFU/ml)
1	Yes	35	45	Yes	3	1.2×10^3
2	Yes	10	50	Yes	2	1.0×10^2
3	Yes	19	45	Yes	2	1.03×10^2

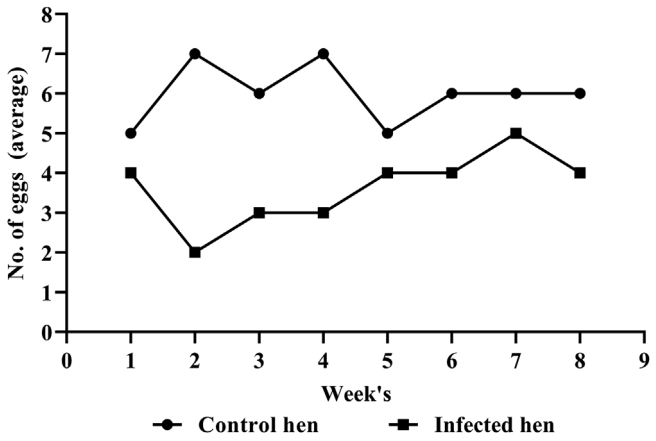

FIGURE 3 Pattern of average egg production by the control group of hens and infected hens over the postinoculation period

of hens showed an average maximum production of six eggs per week, whereas the infected hens laid approximately four eggs per week, indicating a 33.33% reduction in egg production (Figure 3).

3.6 | Identification of *Salmonella* by PCR amplification of the *invA* gene

Detection of the PCR-amplified genus-specific *invA* gene product (284 bp), which was resolved in a 1.5% agarose gel, confirmed the presence of *Salmonella* spp. (Nagappa et al., 2007).

4 | DISCUSSION

The present study was designed to assess the transmission of *S. enteritidis* in different cases, as it is responsible for salmonellosis in humans. In our experiment, *S. enteritidis* was taken under consideration because incidences of egg contamination were found to be higher than other serovars when hens were orally inoculated with *S. enteritidis* (Gast et al., 2014). The models described herein represent horizontal transmission, vertical transmission, and cross-contamination. Moreover, the effect of *S. enteritidis* infection on egg production by laying hens was also observed.

In hen-to-hen transmission, *S. enteritidis* was transmitted from infected hens to in-contact hens. According to this study, 60% transmission was observed within 61 days of postinoculation. Therefore, the calculated transmission rate per day was 0.98%. A study reported that the transmission rate for boiler chickens fed liquid feed was 1.15 per day (Heres et al., 2004). Another simulative study that considered several parameters (initial transmission rate, reproduction number, generation time, etc.) showed that a 92% colonization level of 20,000 laying hens could be possible within 80 days after colonization of the first hen (Thomas et al., 2009). This great discrepancy between our findings and the findings mentioned above might be due to differences in experimental design, sample size, and transmission model to evaluate the transmission characteristics. We also observed that 26.7% transmission occurred within the first month (31 days) of postinoculation, and the transmission increased gradually in later days, which could be attributed to continuous shedding of *S. enteritidis* in faeces and thus more exposure of in-contact hens to this pathogen. In all inoculated hens, the oral dose of *S. enteritidis* was 1×10^9 CFU/ml, and

13 out of 15 hens from the inoculated group shed this pathogenic microbe in faeces. A similar result was also reported by Thomas et al. (2009) using the same oral dose and strain of *S. enteritidis*, where all hens did not show positive cloacal swab samples. Moreover, a review by Cox et al. (2000) showed that the inoculation of hens with 1×10^6 CFU/ml caused internal egg contamination, but not all faecal samples were positive for *Salmonella*. Therefore, the results from experimentally infected hens indicated that faecal shedding depends on the oral dose. In addition to the culture test, we also carried out ELISA to study the serum antibody response in both inoculated and in-contact hens. All inoculated hens, including two hens that were negative in the culture test, showed a positive antibody response. Therefore, a negative culture test of cloacal swab does not always indicate the absence of *Salmonella* infection. Assessment of horizontal transmission using eggshell samples found that 58% of eggs were *S. enteritidis* positive. These eggs can be contaminated from infected reproductive organs or from faeces. A small number of infected hens could increase the likelihood of horizontal transmission to other birds if they were involved in persistent shedding of *S. enteritidis*, leading to contaminated egg production (Gast et al., 2014). Among 87 eggs, 63 corresponded to inoculated hens, which indicated that the occurrence of *Salmonella* was higher in this group compared to in-contact hens. The percentage of eggs laid by hens orally inoculated with *S. enteritidis* and contact-exposed hens was 70.8% and 54.2%, respectively (Gast, 1993). In the vertical transmission experiment, 5.33% of the eggs showed contamination in their internal contents. The adherence ability of *S. enteritidis* to the reproductive mucosa of hens has already been reported (Gast et al., 2019; Keller et al., 1995; Wales & Davies, 2011). A previous study with eggs laid between 5 and 25 days postinoculation by hens showed that 4.22% of the egg contents were contaminated with *S. enteritidis* (Gast et al., 2014). According to Bichler et al. (1996), 2.9% of eggs collected from inoculated hens were internally positive for *S. enteritidis*, where the oral infectious dose was 1×10^{10} CFU/ml per hen. The incidence of *Salmonella* in egg contents was lower than that in the outer shell due to egg membrane barrier protection and the presence of antibacterial compounds in egg albumen. The penetration of eggshell by this pathogen can also contaminate egg contents (De Reu et al., 2006). Although, in our study, we collected eggs, washed them with 70% alcohol, and then refrigerated, but it would not be concluded that egg contents were not contaminated by penetration because this pathogen was observed to be penetrated with the highest incidence between 15 min and 3 h postlaying at 25°C storage temperature (Miyamoto et al., 1998). There is insufficient evidence to establish cross-contamination as an important contributing factor in foodborne outbreaks (Carrasco et al., 2012). Our experiment proved that artificially contaminated containers could be a potential vehicle for the transmission of *Salmonella*. Although the number of *Salmonella* in the container during the cross-contamination experiment was low, cross-contamination (lack of hygiene, contaminated container, etc.) played a vital role in the development of *Salmonella*-associated foodborne outbreaks.

We amplified the *invA* gene because the detection of *Salmonella* by amplifying this gene is now considered an international standard (Malorny et al., 2003). It is a pathogenic inner membrane protein of *Salmonella* used for invading epithelial cells of the host (Darwin & Miller, 1999). In fact, this gene is essential for complete virulence in *Salmonella* to cause salmonellosis by penetrating deeper tissues (El-Sebay et al., 2017). As all the hens were tested before the experiment and sterile conditions were maintained wherever needed, it is highly unlikely that another species of *Salmonella* would invade. *invA*-specific primers were chosen due to their universality in *Salmonella* spp. detection and its ready availability in the laboratory.

We observed the effect of *S. enteritidis* infection on the egg production rate. Egg production was reduced by approximately 33.33% in the infected hen group compared to the control group. It was also found that the egg production rate was higher within the later month of postinoculation than in the first month. However, egg production was always lower in infected hens during the entire experimental period. This finding was similar to the report in which a noticeable decrease in egg production was reported using an oral dose of 1×10^8 CFU/ml per hen (Shivaprasad et al., 1990). Another study conducted by Cox et al. (2000) observed that the egg production rate for infected laying hens remained unaffected when hens were infected with 1×10^6 CFU/ml bacterial culture per hen. Upon comparing these observations with our results, we conclude that the effect of *Salmonella* infection of hens on egg production might be dependent on the experimental dose of inoculation. Inoculation with a higher number of pathogens resulted in reproductive tract infections, which further affected egg production.

The abovementioned models clearly described the transmission ability of *S. enteritidis* in hens and eggs that play a pivotal function in the outbreak of salmonellosis. Analysis of different types of transmission provides us with a useful scientific background to understand the impact of *S. enteritidis* infections in the poultry industry. This study provided more insight into the linkage between faecal shedding of *Salmonella* and its dissemination into other hens and eggs.

5 | CONCLUSION

In conclusion, our study includes vertical and horizontal transmissions of *S. enteritidis* along with the study of cross-contamination and the effect of infection on egg production. To the best of our knowledge, this is the first report that experimentally demonstrated the transmission of *Salmonella* in poultry and poultry products in Bangladesh. The occurrence of *Salmonella* on eggshell and internal contents and a drop in egg production due to its colonization are a considerable threat to both human health and the poultry industry. This study provides an adequate scientific background to develop a surveillance program for eradicating this zoonotic pathogen from poultry and poultry products in Bangladesh and thus reduce economical loss.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

ETHICS STATEMENT

All experiments involving animals were reviewed and approved (Ref. no. 130/Biol. Scs./2021-2022) by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka (approved on 31 July 2021).

AUTHOR CONTRIBUTIONS

Data curation, formal analysis, investigation, methodology, and writing—original draft: Mst Fatema Khatun. Data curation, formal analysis, investigation, methodology, and writing—original draft: Md Abu Sayem Khan. Methodology, validation, and writing—review and editing: Md Firoz Ahmed. Conceptualization, and writing—review and editing: Md Majibur Rahman. Conceptualization, funding acquisition, methodology, supervision, and writing—review and editing: Sabita Rezwana Rahman.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article, as no datasets were generated or analyzed during the current study.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.874

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