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Prevalence and molecular detection of multidrug-resistant *Salmonella* spp. isolated from eggshells in the local markets of Dhaka, Bangladesh

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Abstract

Background and Aim: *Salmonella* spp. are frequently associated with various parts of the egg, including the shell, and cause foodborne outbreaks worldwide. Antibiotic-resistant *Salmonella* spp. pose serious threats to human and animal health; therefore, preventive measures against these pathogens are important. This study aimed to isolate and characterize *Salmonella* spp. from eggshell samples from different local markets in Dhaka, Bangladesh.

Materials and Methods: *Salmonella* spp. were recovered from eggshells by enrichment culture and biochemical tests and characterized through molecular amplification of *Salmonella*-specific genes. Antibiotic sensitivity testing and molecular detection of isolates were performed by disk diffusion method and polymerase chain reaction (PCR), respectively. The *invA, fliC*, and *sdfI* genes were used in PCR to identify the genus *Salmonella*, and the species *Salmonella* Typhimurium and *Salmonella* Enteritidis, respectively.

Results: The prevalence of *Salmonella* spp. was recorded as 40%, in which *S.* Typhimurium was the predominant serotype. PCR analysis revealed that 100%, 59%, and 13.6% of these isolates possessed the *invA, fliC*, and *sdfI* genes, respectively. The isolates exhibited multidrug resistance phenotypes, with resistance (95.5%) toward tetracycline, sulfamethoxazole, and clindamycin and sensitivity (86.3%) toward chloramphenicol.

Conclusion: The findings of this study reflect the potential of eggs as a reservoir of multidrug-resistant *Salmonella* spp.; therefore, we recommend the careful handling of eggs to avoid contamination from farm to market.

Keywords: antibiotic resistance, eggs, health hazards, poultry industry, *Salmonella*.

Introduction

Bacteria in the genus *Salmonella* are recognized as some of the most widespread foodborne pathogens and have a significant global economic impact [1]. *Salmonella* account for 93.8 million gastroenteritis cases a year worldwide, which result in 155,000 deaths each year [2]. In 2000, *Salmonella* spp. was responsible for 22 million enteric fever cases that resulted in 200,000 deaths worldwide, mostly in underdeveloped countries [3]. At present, 2463 serotypes of *Salmonella* have been identified that cause numerous infections in humans and animals [4]. Among them, *Salmonella* Enteritidis is the most frequently (65%) encountered serotype in non-typhoidal salmonellosis cases, followed by *Salmonella* Typhimurium (12%) and *Salmonella* Newport (4%) [5]. Although almost all strains of *Salmonella* are reported to be pathogenic, the severity of infection depends on the serotype and the susceptibility of the host [6]. A wide variety of foods, including meat, eggs, poultry products, and milk, can act as a vehicle for the transmission of *Salmonella* spp., and the consumption of these foods, when contaminated with *Salmonella*, can result in infection, including gastroenteritis and typhoid fever, which constitutes 95% of human cases of salmonellosis [7]. In the European Union in 2010, eggs and egg products were reported as transmission vehicles in 43.7% of cases of human foodborne outbreaks of salmonellosis [8].

Poultry farming is a profitable business worldwide; considering the total GDP of Bangladesh, the contribution from livestock and poultry is estimated to be 1.47% [9]. In Bangladesh, around 20% of the population depends on poultry farming as a primary means of livelihood, and around 50% of the population considers it a part-time employment opportunity [10]. Along with the crisis and instability in the poultry industry, infectious diseases such as salmonellosis and avian influenza play a significant role in the achievement of expected growth in this sector. In Bangladesh, the prevalence of *Salmonella* infections in layer poultry ranges from 28% to 53.2%, suggesting its detrimental effect on the development of the poultry sector [11]. Various *Salmonella* serotypes have been frequently isolated from poultry farms, indicating the potential of the farm environment as a reservoir for *Salmonella*

spp. One possible reason for the higher occurrence of *Salmonella* spp. is poor or inadequate sanitation. This pathogen can occur on eggs through horizontal (from surroundings) or vertical (during egg formation) transmission [12].

Antibiotics are frequently used in poultry farms as growth promoters and to treat bacterial infections in broiler chickens [9]. Indiscriminate use or the overuse of antibiotics can exert selective pressure that leads to the evolution and dissemination of drug-resistant pathogens. According to one study, 300 million human deaths, $100 trillion in financial losses, and an 11% reduction in animal production will occur by 2050 due to the dissemination of antimicrobial resistance [13]. Several studies from Bangladesh have reported that isolates of *Salmonella* recovered from broiler farms (cloaca swabs, eggs, feed, etc.) have become resistant to different antibiotics, including ampicillin, amoxicillin, tetracycline, and chloramphenicol, to varying degrees [9, 14]. The continuous increase and spread of multidrug-resistant pathogens may have devastating consequences to public health, specifically in human morbidity, mortality, hospitalizations, and treatment strategies.

Eggs are a cheap and nutritious food, and, in Bangladesh, their consumption has increased considerably in the past few decades. However, eggs can serve as a potential vehicle for *Salmonella* contamination. In addition, based on the previous reports on the occurrence of antibiotic-resistant *Salmonella* in poultry, it is likely that a considerable proportion of egg associated *Salmonella* has already become multidrug-resistant. Because eggs from various poultry production sites are transported and sold in Dhaka, systematic studies would provide important insights into the antibiotic resistance patterns of *Salmonella* spp. in the poultry production environment in Bangladesh.

This study aimed to estimate the prevalence of antibiotic resistance among *Salmonella* spp. isolated from eggs from various markets in Dhaka, Bangladesh, with special emphasis on *S.* Typhimurium and *S.* Enteritidis.

Materials and Methods

Ethical approval

This study did not require ethical approval.

Study period and location

The study was conducted from January to July 2019. The samples were collected from local markets in Dhaka, Bangladesh and processed at the laboratory of the Department of Microbiology, University of Dhaka, for bacteriological analysis.

Sample collection and processing

A total of 55 eggshell surface swab samples were collected from six open markets in Dhaka city, Bangladesh: Newmarket, Krishi Market (Mohammadpur), Town Hall Market (Mohammadpur), Farmgate, Karwan Bazar, and Anandabazar. The swabs were inoculated into buffered peptone water followed by overnight incubation at 37°C for pre-enrichment. Then, 1 mL of each sample was inoculated in selenite cysteine broth (HiMedia, Mumbai, India) for selective enrichment and incubated again at 37°C for 24 h.

Isolation and presumptive identification of *Salmonella* spp.

The enriched broth was diluted and spread onto xylose lysine deoxycholate (XLD) agar and Salmonella–Shigella (SS) agar (HiMedia, Mumbai, India) and the plates were incubated at 37°C for 24 h. Following incubation, the plates were observed for the presence of *Salmonella*-like colonies. Selected pure colonies were subsequently streaked onto XLD agar to obtain a pure culture of isolates. Various biochemical tests, including catalase, indole, methyl red, Voges–Proskauer, citrate utilization (IMViC), oxidase, motility indole urease, and Kligler's Iron Agar (KIA), were performed for preliminary identification of *Salmonella* spp.

Chromosomal DNA extraction by boil method

Bacterial DNA was extracted following the procedures previously described by De Medici *et al.* [15] with some modifications. In brief, 1 mL of freshly grown culture was centrifuged for 5 min at 10,000× *g*, the supernatant was discarded, and 200 µL of DNase-RNase-free distilled water was added to resuspend the pellet. The culture was then boiled at 100°C for 10 min, then immediately transferred to ice for 10 min. After centrifugation at 12,000× *g* for 5 min, the supernatant was collected carefully, stored at −20°C, and then used as the template DNA in the polymerase chain reaction (PCR) analysis.

Molecular detection of *Salmonella* spp. by PCR

The polymerase chain reaction was used to detect the presence of the *invA*, *sdfI*, and *fliC* genes among the isolates. Each gene was amplified separately. The volume for each reaction was 25 µL, consisting of12.5 µL of 2× Master Mix (Promega, USA), 1 µL of forward primer, 1 µL of reverse primer, 4 µL of template DNA, and 6.5 µL of nuclease-free water. The primer sequences, annealing temperature, and amplicon size are outlined in Table-1 [16–18]. The DNA template of *S.* Typhimurium ATCC 14028 was utilized as the positive control. The PCR products were separated electrophoretically on 1.5% agarose gel (Promega, USA), stained with ethidium bromide, and then visualized and photographed using a UV transilluminator. Both 1 kb and 100 bp ladders (Promega, USA) were used as molecular size markers to determine the position of desired bands.

Determination of antimicrobial susceptibility of *Salmonella* isolates

The isolates' sensitivities to various antibiotics were characterized by Kirby–Bauer disk diffusion methods [19]. The following disks (Oxoid, Basingstoke, United Kingdom) were used:

Table-1: PCR primer sequences and thermocycling conditions used for molecular detection of *Salmonella* spp.

Gene	Primer	Sequence	PCR conditions (initial denaturation, cycle of denaturation-annealing-extension, and final extension)	Product size (bp)	Bacteria	Reference
invA	Forward Reverse	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	1 cycle of 95°C/1 min, 38 cycles of (95°C/30 s, 64°C/30 s, 72°C/30 s), 1 cycle of 72°C/4 min	283	*Salmonella* spp.	[16]
sdfI	Forward Reverse	TGTGTTTTATCTGATGCAAGAGG CGTTCTTCTGGTACTTACGATGAC	1 cycle of 95°C/5 min, 30 cycles of (95°C/30 s, 58°C/30 s,72°C/45 s), 1 cycle of 72°C/4 min	333	*Salmonella* Enteritidis	[17]
fliC	Forward Reverse	CGGTGTTGCCCAGGTTGGTAA ACTGGTAAAGATGGCT	1 cycle of 95°C/5 min, 35 cycles of (95°C/30 s, 58°C/30 s, 72°C/43 s), 1 cycle of 72°C/5 min	620	*Salmonella* Typhimurium	[18]

PCR=Polymerase chain reaction

Chloramphenicol (30 μg), amoxicillin (30 μg), tetracycline (30 μg), nalidixic acid (30 μg), gentamicin (10 μg), sulfamethoxazole (25 μg), azithromycin (15 μg), clindamycin (2 μg), and streptomycin (10 μg). A freshly grown pure colony was inoculated into Mueller-Hinton broth and then incubated at 37°C for 4 h with shaking. The turbidity of the growing cultures was adjusted to McFarland 0.5 standard (2×10^8 Colony-forming unit/mL). To obtain a homogeneous mat of bacterial culture, a sterile cotton swab was dipped into the diluted, standardized solution, and streaked uniformly. Antibiotic disks were then placed onto the inoculated plates with sterile forceps. After a 24 h incubation period at 37°C, the diameters of the zones of inhibition were measured, and the findings were interpreted according to the Clinical and Laboratory Standard Institute guidelines [20]. The number of antibiotics to which isolates were resistant was divided by the number of antibiotics employed in the study to calculate the multiple antibiotic resistance (MAR) index.

Results

Presumptive identification of *Salmonella* spp.

A total of 40 pure colonies with *Salmonella*-like characteristics were selected for biochemical characterization. These isolates produced black colonies on XLD and SS agar as a result of H_2S production, showed negative results in the indole and Voges–Proskauer tests, positive results in the methyl red, citrate, and catalase tests, and indicated dextrose fermentation and hydride sulfide production in the KIA test. Variations were observed in the urease production and motility tests. The isolates (22/55) that showed a negative urease reaction were further considered for molecular characterization and antibiotic sensitivity tests.

Molecular detection of *Salmonella* spp.

All 22 isolates yielded bands at the 284 bp position in gel electrophoresis, which corresponded to the *invA* gene (Figure-1). The *fliC* gene was detected in 13 isolates, while the *sdfI* gene was detected in three isolates, suggesting the presence of *S.* Typhimurium and *S.* Enteritidis in 23.6% and 5.4% of the collected samples, respectively.

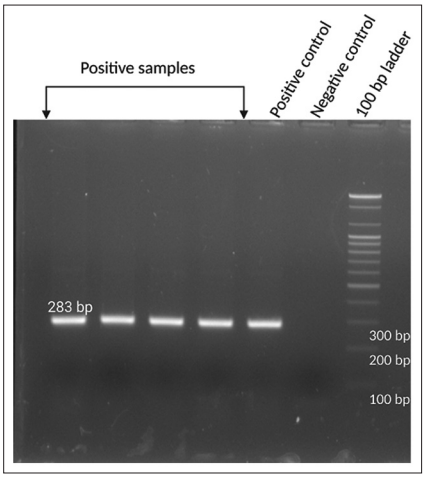

Figure-1: A 283 bp *invA* gene found among all *Salmonella* isolates was resolved in 1.5% agarose gel.

Antibiotic resistance trends

Only one of the 22 isolates was observed to be sensitive to tetracycline, sulfamethoxazole, and clindamycin, while the majority of isolates (95.5%) showed resistance to these antibiotics. The percentage of isolates that showed resistance to nalidixic acid, amoxicillin, and azithromycin was 82%, 64%, and 59%, respectively (Figure-2). Most of the isolates were susceptible to the action of chloramphenicol (86.3%), streptomycin (63.6%), and gentamicin (59%). Nine (41%) isolates showed sensitivity to at least two antibiotics (streptomycin and chloramphenicol).

Discussion

The present study was conducted to characterize the prevalence of multidrug-resistant *Salmonella* spp. among eggshells from markets in Dhaka, Bangladesh. The overall prevalence of *Salmonella* spp. in collected

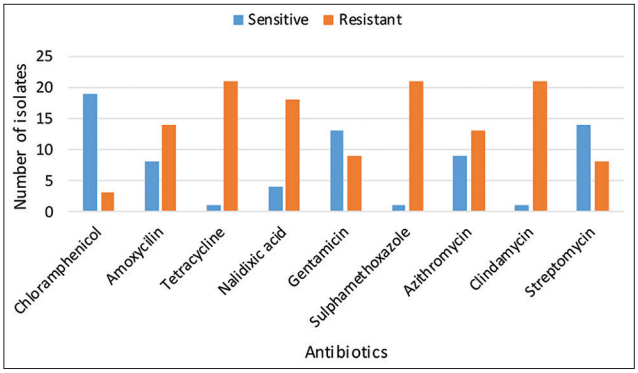

Figure-2: Antibiotic resistance profile of *Salmonella* spp.

samples was estimated to be 40%. This finding is similar to another study, which reported a 45.8% prevalence of *Salmonella* on eggshell surfaces from Chittagong, Bangladesh [21]. The scenario was different in 2010, when the occurrence of *Salmonella* spp. on eggshells from various markets in Dhaka city was reported to be 6% [22]. Such a rapid increase in drug resistance can be attributed to eggshell surface contamination during processing, storage, and transportation [23]. The prevalence of *Salmonell*a spp. on eggshells has been reported to be 83% in samples collected from Savar, Bangladesh (2011-12), and 28% in those collected from Dhaka (2014-15) [24, 25], suggesting that the prevalence of *Salmonella* spp. on eggshells varies depending on the sampling site.

Conventional methods of the isolation of bacterial pathogens focus on phenotypic characteristics, including their cultural properties on selective media and their ability to utilize different substrates through numerous biochemical tests. Other than *Salmonella*, bacterial species such as *Citrobacter* and *Proteus* can produce black colonies on XLD agar [26, 27]. Even after selective isolation, *Salmonella* spp. colonies can be contaminated by *Proteus* swarm cells, making *Salmonella* recovery and biochemical identification challenging due to the presence of atypical biochemical profiles that typically match with *Proteus* biochemical profiles [28]. For proper identification of *Salmonella* spp., we subjected isolates to a wide array of biochemical tests, in which all isolates displayed the same biochemical trends, except for urease formation and motility. The urease test acts as the differentiating tool between *Salmonella* and *Proteus*, where *Salmonella* is urea negative and *Proteus* is urea positive [29]. Although conventional methods of detection provide phenotypic insights into bacteria, they are time-consuming and laborious. Consequently, molecular techniques have been developed that make the identification of bacteria accurate, fast, and reliable. The PCR is one such molecular technique for the rapid, reliable detection, and identification of foodborne pathogens. Amplification of the *invA* gene is currently accepted as an international standard for fast detection of the *Salmonella* genus [30]. This virulence gene produces a protein in the inner membrane of bacteria that accelerate the invasion to the host's epithelial cells [31]. The PCR results confirmed the presence of *Salmonella* in the amplification of 284 bp DNA fragments for the *invA* gene in all 22 presumptively identified *Salmonella* isolates**,** irrespective of serovar. This finding was supported by other studies that also found that all *Salmonella* spp. were positive for *invA* amplification [32, 33]. We could conclude that all of the isolates were pathogenic as they carried the *invA* gene, and were, therefore, capable of causing salmonellosis if introduced to the human body. Epidemiological research also indicates that the presence of *Salmonella* in poultry products is linked to the prevalence of salmonellosis in people [34]. Amplification of the *sdfI* gene indicated that 3 of the 22 isolates (13.7%) were *S.* Enteritidis. This finding is in agreement with another study that identified six out of 67 *invA*-positive *Salmonella* spp. as *S.* Enteritidis using the same primer [35]. *sdfI* was found to be unique to *S.* Enteritidis isolated using the suppression subtractive hybridization method [17]. As this fragment is absent in other *Salmonella* spp., it can be used as a diagnostic marker for the identification of *S.* Enteritidis**.** To differentiate *S.* Enteritidis from its close relatives *Salmonella* Dublin and *Salmonella pullorum*, Agron *et al.* [17] utilized a *sdfI* primer that successfully distinguished 33 known *S.* Enteritidis isolates. *fliC* is the flagellin gene that encodes the major structural component of the flagella in *S.* Typhimurium [36]. In this study, 13/22 (59%) isolates were confirmed as *S.* Typhimurium in the amplification of the *fliC* gene, making it the dominant serotype among all isolates. Numerous studies have utilized the amplification of this gene to distinguish *S.* Typhimurium from other serotypes of *Salmonella* [37, 38]. Singh *et al*. [23] and Li *et al*. [39] have also reported the predominance of *S.* Typhimurium among eggshell samples. The molecular amplification of species-specific genes is, therefore, a reliable tool for the confirmed detection of *Salmonella* spp., which is necessary for protecting public health and improving the quality of poultry products.

In the antibiotic susceptibility tests, we used nine antibiotics, representing eight different classes of antibiotic (chloramphenicol, penicillin, tetracycline, quinolone, aminoglycoside, macrolide, lincosamide, and aminoglycoside) to observe the current status of antibiotic resistance among *Salmonella* spp. isolated from eggs. We found that the highest sensitivity was to chloramphenicol (86.3%), whereas maximum resistance was recorded toward tetracycline, sulfamethoxazole, and clindamycin. Mahmud *et al*. [21] reported that 95.5% of *Salmonella* spp. recovered from eggshells were resistant to tetracycline. Recent studies from Bangladesh found that egg-associated *Salmonella* spp. were susceptible toward the action of gentamicin and amoxicillin at varying percentages [40, 41]. Our findings are supported by a study from Ethiopia, which observed the same sensitivity

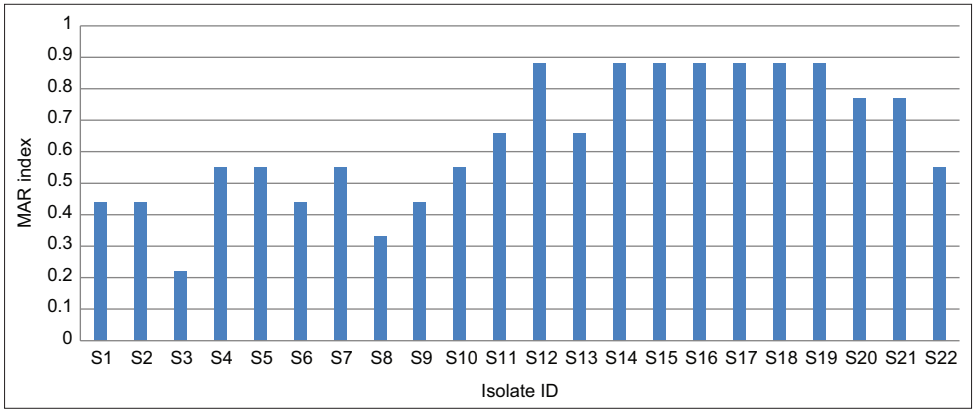

Figure-3: Multiple antibiotic resistance index of *Salmonella* spp. isolates.

toward chloramphenicol [42]. Alarmingly, this study also detected multiple drug-resistant *Salmonella* spp. Seven isolates were resistant to eight antibiotics (MAR index: 0.88) and two isolates were resistant to seven and six antibiotics, respectively (MAR index: 0.77 and 0.66) (Figure-3). The occurrence of multidrug-resistant *Salmonella* spp. isolated from eggs is evident in the literature [19,43], and multidrug-resistant bacteria are frequently isolated from poultry samples in Bangladesh [9, 11, 44]. The dramatic increase in the prevalence of multiple antibiotic-resistant foodborne pathogens is probably linked to the indiscriminate and widespread use of antimicrobial agents in veterinary and human medicine [45].

Limitations of the study

The key limitations of our study are the small sample size and the lack of sequencing data. Although we confirmed the isolation of *S.* Enteritidis and *S.* Typhimurium through amplification of the species-specific *sdfI* and *fliC* genes, the addition of sequencing is necessary for the validation of these results. However, despite these limitations, this study provides important information regarding the presence, characterization, and detection of *Salmonella* spp. from eggshells and has enormous public health significance.

Conclusion

This study was focused on the phenotypic detection of antibiotic sensitivity among *Salmonella* spp. isolated from eggshells in Dhaka, Bangladesh, and their subsequent detection by genotypic methods to provide an accurate profile of ongoing antibiotic resistance trends and to add to the understanding of the harmful consequences of transmission to humans. We showed that PCR-based detection of species-specific genes could be a valuable strategy in the identification of pathogens, which is vital for the selection of the treatment of infection. Novel studies into bacteriophage therapy, the generation of new antibiotics, and the rapid identification of pathogens are needed to protect public health from the infections caused by multidrug-resistant *Salmonella* spp. Our findings suggest that there are knowledge gaps in the current safety and hygiene practices in the poultry industry in Bangladesh. Therefore, we recommend the intervention of appropriate strategies to minimize the occurrence of *Salmonella* spp. in the food chain to protect public health.

Authors' Contributions

SRR: Conceptualized and designed the study. TH and MASK: Performed laboratory experiments. MASK and MFA: Conducted data analysis and wrote the manuscript. All authors have read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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