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Research Article

Salmonella enterica Serovar Typhimurium and Enteritidis Isolated from Raw Shrimp in Bangladesh: An Investigation Based on Molecular Characteristics, Survival, Virulence, Antibiotic Resistance, and Biofilm Formation Attributes

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Shrimp is the white gold of Bangladesh, with the second-highest income source from exporting to foreign countries. Contamination with *Salmonella* spp. is now one of the significant issues for Bangladesh to export. Proper characterization of the salmonella pathogen is thus necessary to avoid undesirable losses due to the rejection of exported shrimp. In Bangladesh, the present condition of raw shrimp contamination with pathogenic *Salmonella* serovars and their survival/virulence properties was not adequately characterized. In this study, we collected 43 raw shrimps as samples from different farms in Jashore, Khulna, and Sathkhira regions. We then maintained standard cultural and biochemical protocols for isolating *Salmonella* strains, followed by the molecular identification of particular *Salmonella* serovars. The standard method for checking its credibility to form biofilm in 0–10% NaCl, tolerate acid/bile stress likewise in the gastrointestinal tract, and resist antimicrobial pressure was performed individually with the particular pathogenic strains. Our results successfully identified eleven *Salmonella* strains with three typhimurium serovars and three enteritidis serovars, which have biofilm-forming capability up to 4–8% NaCl, acid/bile habituation alike stomach/small intestine of humans, and resistance against necessary antibiotics generally used in treating human and poultry infection signifying the impending danger in the shrimp industry. While previous studies of Bangladesh successfully isolated *Salmonella* only presumptively, our research focused mainly on molecular characterization of the human *Salmonella* pathogen along with important survival and virulent attributes, such as biofilm formation, acid/bile tolerance, and antibiotic resistance of selected *S.* typhimurium and *S.* enteritidis strains. Further study with more sampling will be necessary to confer the transmission route of the pathogen from the natural reservoir to the shrimp industry.

1. Introduction

Food safety is now a significant public health concern that occurs due to food-borne pathogenicity, causing hospitalization, morbidity, and even mortality after consuming different food items, including shrimp and ready-to-eat shrimp products. Shrimp has a high translational value in the international fishery trade [1], and worldwide consumption of this crustacean is increasing. However, continuous reports on contamination with food-borne

pathogens are noticed as they are frequently consumed after light cooking or even raw, especially in off-home services [2–7].

Among these pathogens, *Salmonella* species—Gram-negative, motile, and nonspore-forming rods belonging to the *Enterobacteriaceae* family—pose the greater danger with their serotype variations and multifaceted defense mechanisms such as biofilm formation, acid/bile tolerance, thriving in desiccated conditions, antibiotic resistance, and others typical survival techniques of bacteria [8–11]. Being one of the most significant enteric pathogens, *Salmonella* is responsible for more than 93 million enteric infections and 155,000 associated deaths annually by causing severe human diseases [12]. Although predominant reservoirs of *Salmonella* spp. are animals and their products, including poultry meat and egg, increasing concern over the waterborne aspect of some pathogenic serovars of this bacterium has been observed in recent reports. These may include different reservoirs, such as fecal-contaminated water, fish feed, birds, amphibians, and reptiles [13–15].

For preliminary molecular screening of all *Salmonella* spp., invasion-related virulence gene invasion A (*invA*) can be used as a universal tool [16]. STM4497 encodes a putative cytoplasmic protein conserved for *Salmonella* typhimurium, and STM3098 produces a putative transcriptional regulator specific for *Salmonella* enteritidis. Thus, these gene targets can be used for serovar differentiation [17].

In nature, many bacteria, including *Salmonella*, are organized in biofilms adhering to abiotic or biotic surfaces and produce an extracellular polymeric matrix that shelters bacteria against adverse environmental conditions [18–20]. The significance of human gastric fluid is well recognized for its innate antibacterial protection against enteric pathogens, including *Salmonella*, especially against *Salmonella enterica* strains. *Salmonella* intrinsically shows resistance to bile and, interestingly, utilizes bile to control gene expression and virulence [21, 22]. More specifically, *Salmonella enterica* serovar typhimurium shows acid tolerance to survive in simulated gastric fluid (SGF) through using its adaptive acid tolerance response (ATR) [23] and activating the PhoP-PhoQ (PhoPQ) two-component regulatory system makes *Salmonella* typhimurium resistant to bile salt [24]. For a long time, antibiotics have been commonly used to prevent disease outbreaks in shrimp farming [25], but the extensive and infrequent dose in shrimp culture might help develop antibiotic-resistant pathogens [26, 27]. Significantly, the emergence of several antibiotic-resistant *Salmonella* serovars using shrimp as a vehicle causes significant morbidity and mortality in humans [28]. Mutation in antibiotic-target genes, overexpression of efflux pumps, increased lipopolysaccharide (LPS) component of the outer cell membrane, downregulation of membrane porins, quorum sensing, and biofilm formation are responsible for antibiotic resistance to *Salmonella* [29].

In Bangladesh, only a few studies were conducted to identify *Salmonella* spp., using merely biochemical analyses from shrimps of markets and farms [30, 31]; however, a report on detecting *Salmonella* in the sediment of a shrimp farm applying both biochemical and PCR assays was also published [32]. Considering these issues, the present study aims to characterize pathogenic *Salmonella* spp. from different farms′ shrimps using biochemical and more specific molecular techniques to identify pathogenic serovars. In addition, a biofilm formation test at different salt concentrations, an assay of survival strength on bile salt and stomach acidic pH of identified *Salmonella* serovars, and finally an antibiotic susceptibility assay were performed to assess the potential of the pathogen to thrive effectively under the conditions usually present in humans.

2. Materials and Methods

2.1. Isolation and Identification of Bacterial Isolates. In July 2018, Bagda shrimp samples ($n = 23$) were purchased from shrimp farms of Chasra, Jashore ($n = 13$), and Jhaudanga, Satkhira ($n = 11$), and in December 2019, Bagda and Golda shrimp samples ($n = 20$) were bought from shrimp farms of Paikgacha, Khulna ($n = 10$), and Tala, Satkhira ($n = 10$). Immediately after collection, all samples were transported to the General Microbiology Laboratory of Jashore University of Science and Technology through an icebox. Upon arrival, 10 grams of each sample (shrimp gills, intestine parts, and flesh) was mixed in 100 ml distilled water (1 : 10), followed by homogenization for two minutes on the stomacher (Seward Stomacher 400 Circulator, UK). We pre-enriched the homogenized samples in the buffer peptone water and tetrathionate broth within three to five hours of collection to avoid unpredictable changes. After culture enrichment, we performed streaking on xylose lysine deoxycholate (XLD) and *Salmonella-Shigella* (SS) agar following Gram staining. Suspected *Salmonella* colonies were selected for biochemical tests (motility test, citrate test, catalase test, Kligler iron agar (KIA) test, methyl red test, Voges–Proskauer test, and urease test) based on Bergey′s Manual of Systematic Bacteriology [33].

2.2. Presence of invA, STM4497, and STM3098 Genes. Twenty-three presumptive *Salmonella* isolates (based on the biochemical tests) were subjected to PCR-based identification targeting the invA gene (forward primer, 5′-GTGAAATTATCGCCACGTTCGGGCAA-3′ and reverse primer, 5′-TCATCGCACC GTCAAAGGAACC-3′) [16], and based on a positive result, we checked the presence of the typhimurium-specific STM4497 gene (forward primer, 5′-AACAA CGGCT CCGGT AATGAGATTG-3′ and reverse primer, 5′-ATGAC AAACT CTTGA TTCTGAAGAT CG-3′) [17] and the enteritidis specific STM3098 gene (forward primer, 5′- TTTGGCGGCGCAGGCGATTC-3′ and reverse primer, 5′-GCCTCCGCCTCATCAATCCG-3′) [17]by following the methodology of Park et al., (2009) [34]; identified *Salmonella* typhimurium and *Salmonella* enteritidis were used in the following assays.

2.3. Assays for Biofilm Formation at Different NaCl Concentrations, Acid and Bile Salt Tolerance, and Survival Ability in Gastric Juice. Using the ring test, the assay of the biofilm formation assay was carried out using *S.* typhimurium

strains (SP6, G-2B0D, and G-3A1F) and *S.* enteritidis strains (B–3B*D, G-3A1D, and G-3A1E) adjusted at different concentrations (0%, 2%, 4%, 6%, 8%, and 10%) of NaCl incubated at 37°C for seven days. After incubation, we used 0.1% crystal violet (before applying crystal violet, the broth was discarded) in the test tubes for 15 minutes at room temperature and the tubes were gently rotated to ensure uniform staining followed by observation of biofilm formation.

To check the survival ability of isolated *Salmonella* typhimurium and *Salmonella* enteritidis under highly acidic conditions, which considered stomach pH~3.5, test tubes containing tryptic soy broth (TSB) were adjusted to different pH (1.5 and 3.5) by adding HCl. After sterilization, we inoculated each test tube with a 1% (v/v) fresh overnight culture of isolated bacteria and incubated it at 37°C for 24 hours. After 24 hours of incubation, their growth was determined by measuring absorbance at 600 nm at one-hour interval up to 4 hours.

TSB broths with different concentrations (2%, 3%, 4%) of bile oxgall (Liofilchem, Italy) were used to determine the tolerance and growth rate of isolated *Salmonella* typhimurium and *Salmonella* enteritidis in bile salt. We adjusted the final pH of the medium to 6.5 and autoclaved it at 121°C. Then, a 1% overnight culture of nutrient broth was inoculated into the TSB broth medium and incubated at 37°C for 24 h, followed by a determination of their growth as per the previous method.

Simulated gastric fluids (SGF) were prepared with pepsin (3.2 g/1000 ml) and NaCl (2 g/1000 ml) and adjusted to pH = 1 by adding HCl. Autoclaved SGF was inoculated with the 1% overnight culture and incubated at 37°C for 24 h. Afterward, we followed the aforementioned protocol.

2.4. Electron Microscopy of Biofilm. Biofilm formation of one of the representative *Salmonella* typhimurium (strain SP6) was observed using scanning electron microscopy (SEM) (ZEISS, Sigma 300). After incubation of the bacteria for 72 h on a falcon tube containing 5 ml of nutrient broth (NB) with different concentrations of NaCl, the broth was discarded, the tubes were rinsed three times with phosphate-buffered saline (PBS; pH 7.2), and the biofilm-forming area was cut into a small piece sized to the carbon tape that was later placed upon the sample holder. The small piece was put into the microcentrifuge tube, and the adhered cells attached to the falcon piece were fixed in 4% glutaraldehyde in PBS for 2 h and then washed three times for 15 min with PBS. Fixed cells were serially treated with ethanol (50% for 15 min, 60% for 15 min, 70% for 15 min, 80% for 15 min, 90% for 15 min, and 100% twice for 15 min each) and successively dehydrated by soaking in 33, 50, 66, and 100% hexamethyldisilazane in ethanol for 15 min each. Dehydrated samples were coated with gold-palladium and examined under the scanning electron microscope [34].

2.5. Antimicrobial Susceptibility Tests. Using the Kirby–Bauer method [35], we tested antimicrobial susceptibility against 12 antibiotics which were as follows: ampicillin (10 μg), amikacin (30 μg), tetracycline (30 μg), gentamycin (10 μg), erythromycin (15 μg), moxifloxacin (5 μg), kanamycin (30 μg), imipenem (10 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), ceftriaxone (30 μg), and colistin (10 μg) as per the recommendation of the CLSI document M100-S23 (M02-A11).

2.6. Statistical Analysis. Graphs were generated in *R* Studio using packages: readxl, tidyverse, ggplot2, ggpubr, and broom.

3. Results

In this study, we first screened out *Salmonella* typhimurium and *Salmonella* enteritidis, a significant health concern for poultry and shrimp industries, from initial raw samples, using cultural, biochemical, and molecular assays. We then performed different assays to check other characteristics, such as biofilm formation capacity, acid and bile salt tolerance, gastric juice resistance, and antibiotic resistance.

3.1. Colony Morphology and Biochemical Identification. The collected samples were taken under standard microbiological procedures first to culture all the bacteria within the sample and then observe the colony characteristics of the cultured bacteria. According to colony morphology (e.g., shape, size, and color) and Gram staining, *Salmonella* spp. was presumptively detected in 23 out of 43 samples. Fifteen isolates among those 23 presumptive-positive samples were biochemically confirmed as *Salmonella* spp (Table S1).

3.2. Molecular Identification. We then confirmed 11 (S3 g, SP6, B–3B*D, B–3B*D2, B–3B*E, G-3B0D, G-2B0E, B–1C1F, G-3A1D, G-3A1E, and G-3A1F) isolates as *Salmonella* spp. by PCR detection of the *inv*A gene (284 bp amplicon) (Figure S1) of 15 isolates that were previously identified as *Salmonella* by biochemical and microbiological tests. From that 11 *Salmonella* spp., 310 bp fragment of the typhimurium-specific (STM4497) gene was amplified (Figure S2) in three isolates (SP6, G-2B0D, and G-3A1F). From the rest of the *inv*A positive isolates, 423 bp fragment of the enteritidis-specific (STM3098) gene was amplified (Figure S3) in three of them (B–3B*D, G-3A1D, and G-3A1E).

3.3. Biofilm Formation. In this study, biofilm formation was observed under different salt concentrations. The range was within 0–10% according to 0%, 2%, 4%, 6%, 8%, and 10%. The *Salmonella* typhimurium strain SP6 formed biofilms on glassware up to 4% salt but failed to form biofilms in salt concentrations greater than 4%. The *S.* typhimurium strains (G-2B0D and G-3A1F) and *S.* enteritidis strains (G-3A1D and G-3A1E) formed biofilms up to 6% salt, while the *S.* enteritidis strain B–3B*D formed a biofilm up to 8% salt concentration (Figure S4 and Table S2).

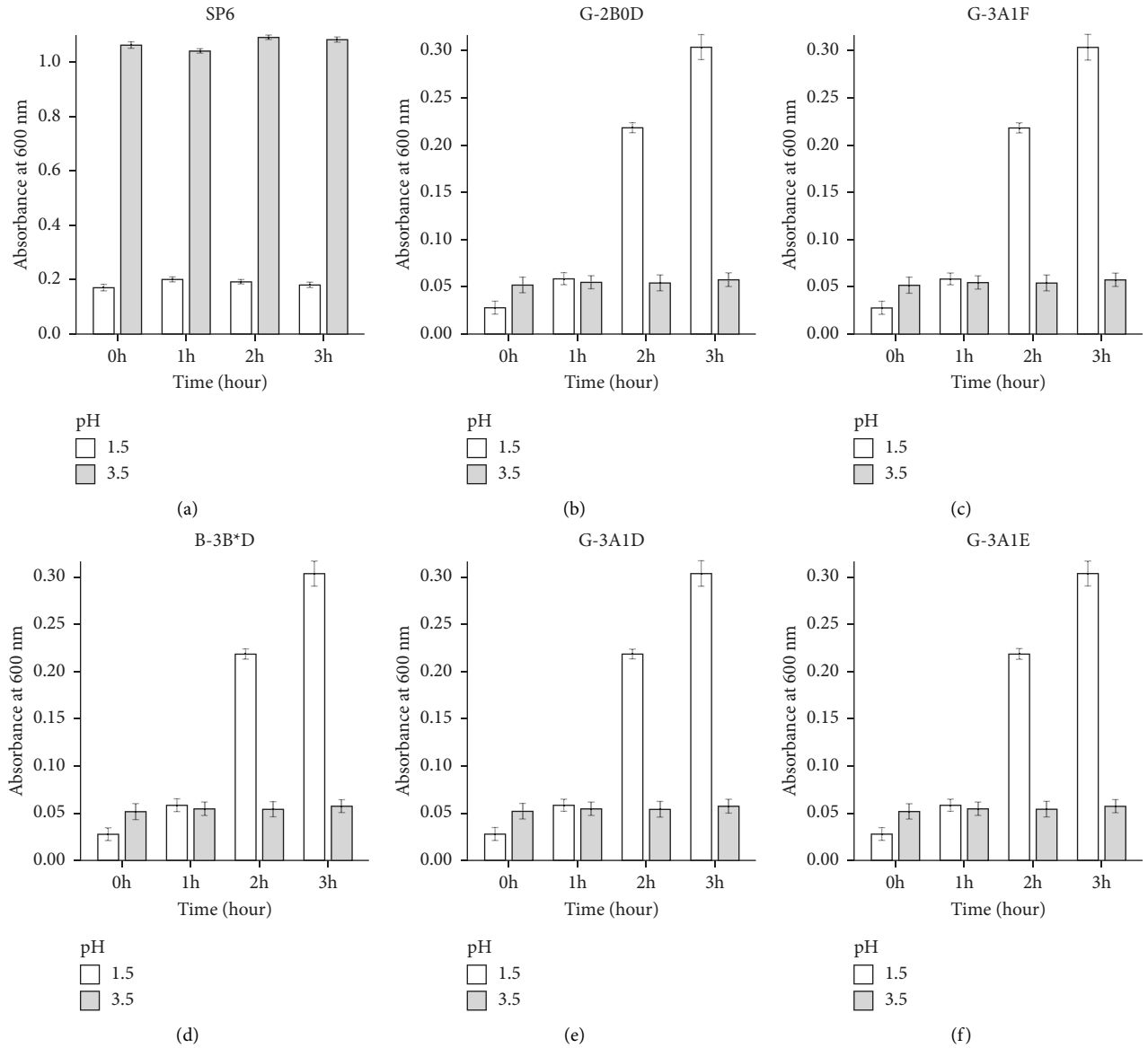

Figure 1: Growth rates of *Salmonella* typhimurium strains (a) SP6, (b) G-2B0D, (c) G-3A1F and *Salmonella* enteritidis strains (d) B–3B*D, (e) G-3A1D, and (f) G-3A1E under acidic conditions (pH 1.5 and pH 3.5). The bars represent the standard deviation of the mean. The graph is generated in RStudio.

3.4. Acid, Bile Salt Tolerance Test, and Survival Ability in Gastric Juice. The SP6 strain survived under high acidic conditions, but growth rates were relatively high at pH 3.5. *S.* Typhimurium strains (G-2B0D and G-3A1F) and *S.* enteritidis strains (B–3B*D and G-3A1E) increased their survival ability with time at pH 1.5 but reduced survival capacity with time at pH 3.5 (Figure 1).

Strains SP6, G-3A1D, G-3A1E, and G-3A1F showed maximum growth at 2% bile salt; however, strain SP6 increased at 3% and 4% bile salt despite less than that in 2% bile salt. At 2% bile salt, strains G-3A1E and G-3A1F increased their survival ability with time. Their growth remained steady in 3% bile salt, while in 4% bile salt, their growth decreased with time (Figure 2).

These strains could easily survive in gastric juice, and with increasing time, the growth rates remained steady. We showed that the growth rates of *Salmonella* typhimurium and *Salmonella* enteritidis increased in simulated gastric fluid but only up to 1 hour (Figure 3).

3.5. Electron Microscopy of Biofilm. Biofilm formation of the representative *Salmonella* enteritidis strain (B–3B*D) at 4% salt concentration was observed under scanning electron microscopy (Figure 4), where characteristic aggregation after biofilm formation was visualized.

3.6. Antibiotic Susceptibility of S. Typhimurium and S. Enteritidis. According to the antibiogram pattern, the isolates were multidrug-resistant (MDR) (Table S3). All isolates had complete resistance to erythromycin and intermediate resistance to ciprofloxacin. *S.* enteritidis strains B–3B*D and

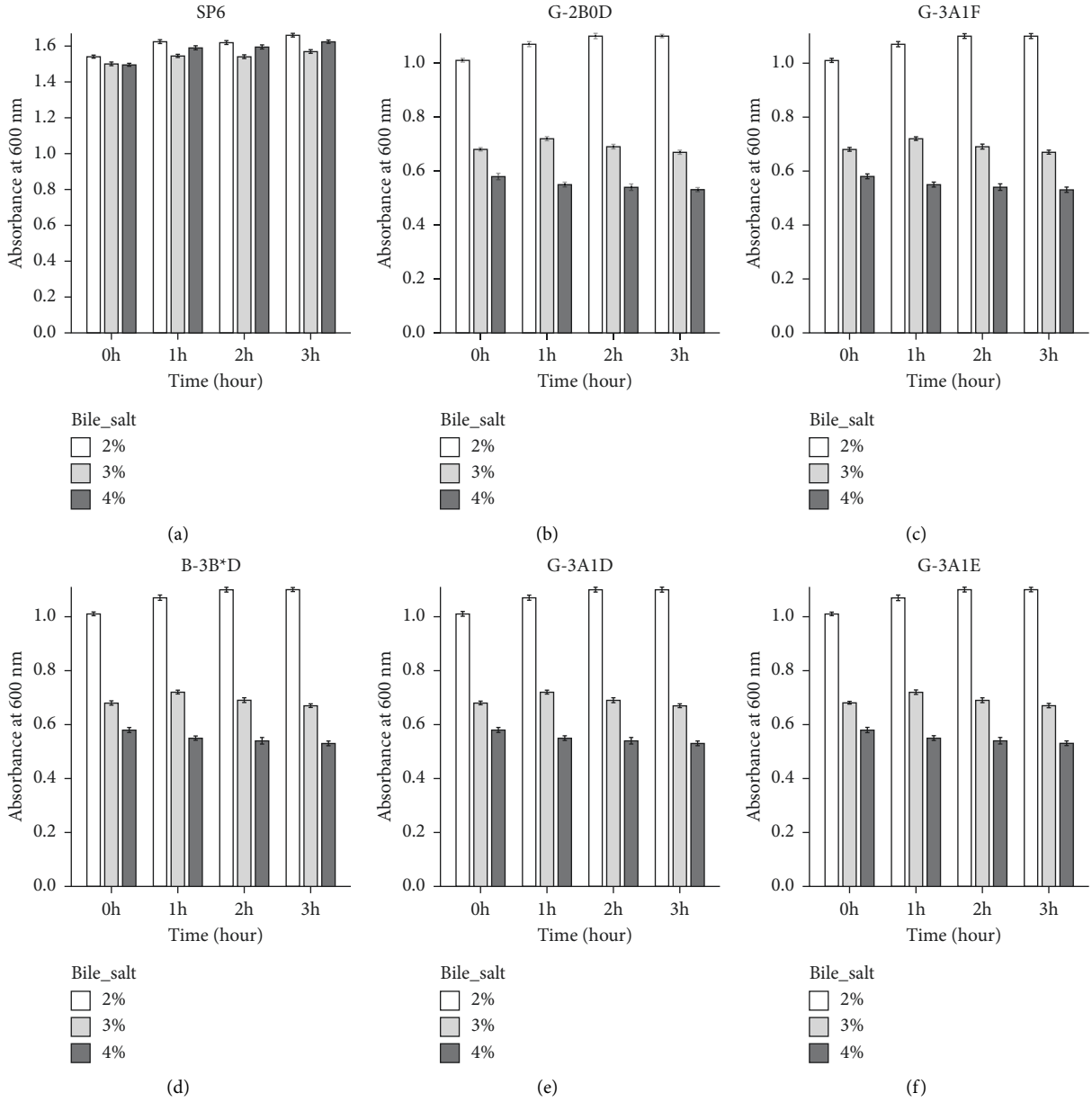

Figure 2: Growth rates of *Salmonella* typhimurium strains (a) SP6, (b) G-2B0D, (c) G-3A1F and *Salmonella* enteritidis strains (d) B–3B*D, (e) G-3A1D, and (f) G-3A1E in bile salt. The bars represent the standard deviation of the mean. The graph is generated in RStudio.

G-3A1E were fully resistant to kanamycin. The rest of the strains were immediately resistant to kanamycin. The *S.* typhimurium strain SP6 showed resistance to amikacin and colistin. Interestingly, this isolate also showed intermediate resistance to kanamycin and ciprofloxacin, which can pose a serious threat to people.

4. Discussion

Shrimp and various shrimp products (processed, frozen, ready-to-cook, and ready-to-eat) significantly impact Bangladesh′s economic status. Our country shows great potential to keep pace with the growing global demand by exporting 40726 tons of shrimp in 2015–16 and earned around US $410 million during the fiscal year (FY) 2017–18 [1, 6]. However, it is a matter of great concern when shrimp products get rejected because of contamination by pathogens such as *Salmonella,* as recently observed in India and China (http://www.shrimpalliance.com/).

For the first time in Bangladesh, we successfully performed molecular characterization of *Salmonella* spp. pathogenic serovars (i.e., typhimurium and enteritidis) directly from raw shrimp, which were found in~14% (six positives among 43) of the samples. A similar study in Egypt detected *Salmonella* in 9.8% of the total shrimp samples collected from different farms and markets [36]. We then identified *Salmonella* typhimurium and *Salmonella* enteritidis strains showing biofilm-forming ability, which might

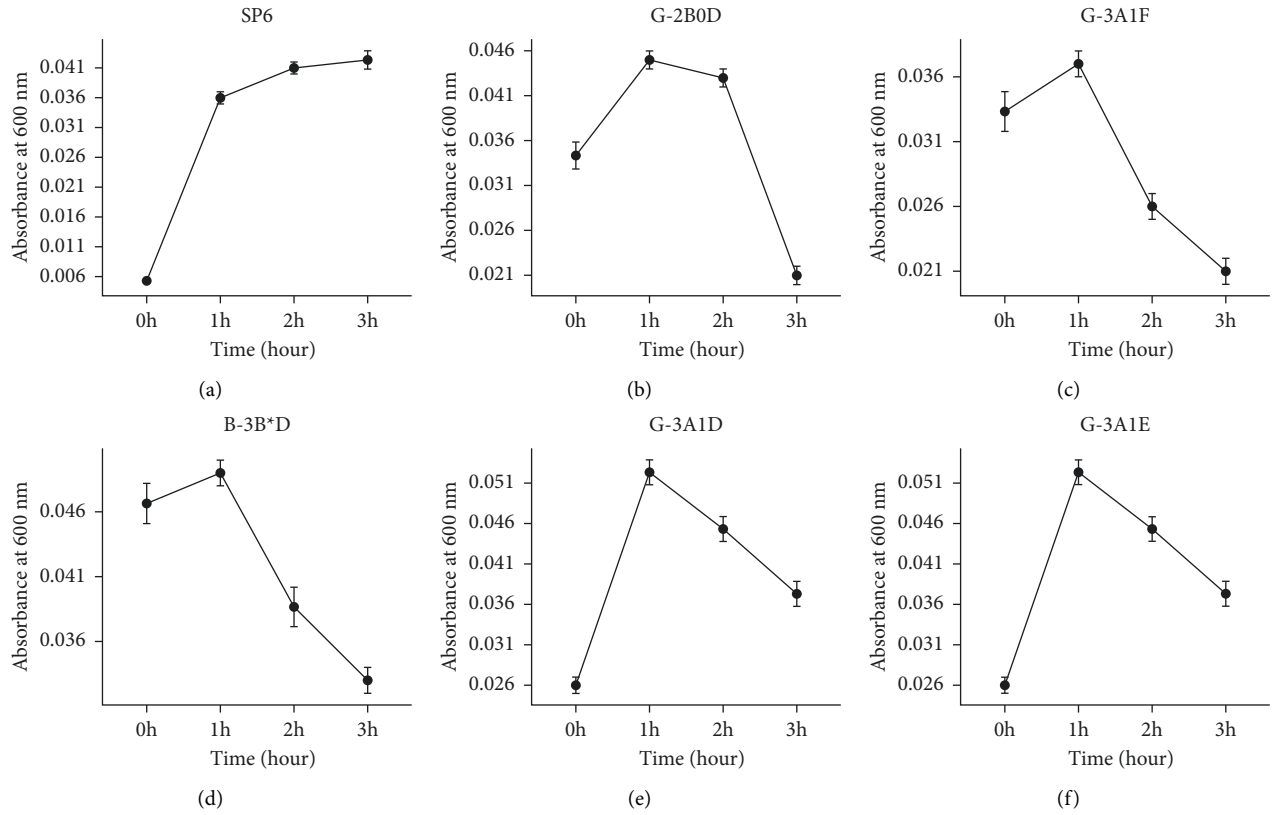

Figure 3: Growth rates of *Salmonella* typhimurium strains (a) SP6, (b) G-2B0D, (c) G-3A1F and *Salmonella* enteritidis strains (d) B–3B*D, (e) G-3A1D, and (f) G-3A1E in simulated gastric juice. The bars represent the standard deviation of the mean. The graph is generated in RStudio.

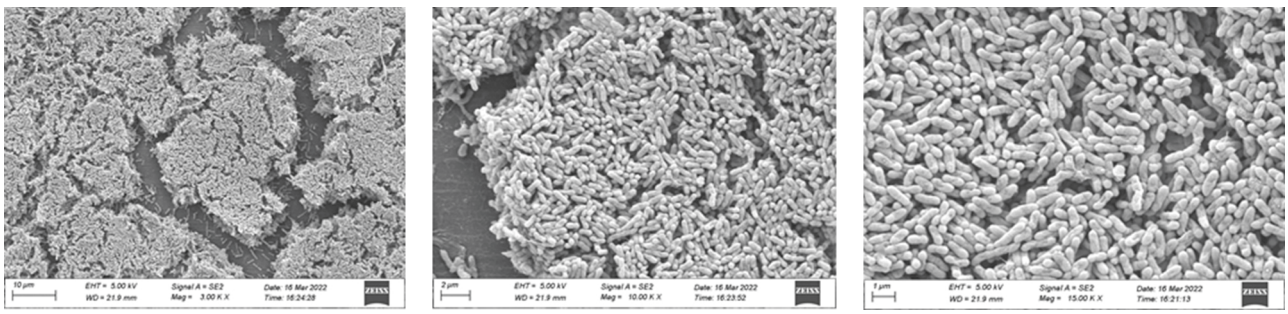

Figure 4: Scanning electron microscopic (SEM) images of the biofilm of the representative *Salmonella* typhimurium strain SP6 formed at 4%(a; b; c) salt concentration. Magnification: (a) 3000x, (b) 10000x, and (c) 15000x; inset bar is representative of (a) 10 μm, (b) 2 μm, and (c) 1 μm.

be crucial for their survival in aquatic and host-specific conditions. Moreover, acid or bile tolerance capacity of these strains may help survive and grow in stomach and intestine of humans to cause infections.

Beshiru et al. (2018) revealed that the biofilm potential of *Salmonella* species could change at different temperatures, nutrient, and incubation periods, and *S.* typhimurium did form biofilms at NaCl concentrations as high as 10% [36]. However, relatively low salt concentrations will stimulate aggregated growth of microorganisms, while higher concentrations inhibit them [37]. In our study, the S. typhimurium strain SP6 could form biofilms at 4% NaCl concentration but failed to do above this concentration. Thus, it can be interpreted that this strain is less adapted to NaCl during biofilm formation. Other strains of S. typhimurium and enteritidis also showed similar responses in the presence of higher NaCl concentrations.

Bile plays a crucial role in expressing different genes associated with pathogenesis, motility, and metabolism in *Salmonella* [9]. *Salmonella* cells that have adapted to bile may have higher chances of survival in the liver, especially in the gallbladder, where the concentration of bile salts is

exceptionally high [38]. This study investigated the survival of *Salmonella* strains in bile, a gastric secretion with antimicrobial activity due to bile salts. *Salmonella* typhimurium and *Salmonella* enteritidis strains exposed to 2% bile salt, which is too high than gastric bile (0.3%) of humans, showed higher growth than 3% and 4% but could survive up to 4% bile salt. Generally, *Salmonella* typhimurium demonstrates particularly high resistance to bile (minimal bactericidal concentrations: >60%), which exceeds bile concentrations encountered during infection [39].

Ingested *Salmonella* spp. pass through the acid barrier of the stomach. Still, most strains of *Salmonella* survive poorly at normal gastric pH (<1.5) but survive well at pH ≥ 4.0. Thus, they may have an adaptive acid tolerance response that might promote survival at lower pH [40]. In survival tests at pH 1.5 and 3.5, the *Salmonella* typhimurium strain SP6 and the *S.* enteritidis strain G-3A1E adapted under high acidic conditions, but maximum growths occurred at pH 3.5 than at pH 1.5. On the contrary, the strains of S. typhimurium (G-2B0D and G-3A1F) and *S.* enteritidis strains (G-3A1D and B–3B*D) adapted under high acidic conditions; hence maximum growths occurred at pH 1.5 than at pH 3.5. Berk et al. (2005) presented a significant variation in acid tolerance response among *S.* typhimurium strains [41]. This resistance confers protection against the stomach′s acidic environment with eventual alterations in virulence.

Significantly, aberrant use of antibiotics and releasing those into the aquatic system are now a significant issue for humanity. Because the pathogen is increasingly becoming resistant to those antibiotics and making the situation worse, antibiotics stay longer in that environment without biodegrading through binding to sediment particles [15]. Zhang et al. (2015) reported resistance chronology of *Salmonella,* sulfonamides > tetracycline > streptomycin > gentamicin > ciprofloxacin > ceftiofur > cefotaxime > ceftazidime > cefepime [42],and, among which ciprofloxacin is usually used for treating human *Salmonella* infections [43]. Although the SP6 strain was not resistant to most antibiotics used in this study, resistance to amikacin, bacitracin, vancomycin, and colistin was not reported previously from the shrimp culture. Thus, this might have a crucial interrelationship with other possible contamination sources in Bangladesh, especially poultry and humans.

The in vivo scenario for the strain SP6, where multiple variables generally come into play during pathogenesis, could be pretty different from that observed in in vitro, simultaneously optimizing one/two parameters. For example, González et al. (2019) speculated that induction of biofilm formation depends on human bile through the expression of different proteins such as curli fimbriae and O-antigen capsule of *Salmonella* [43]. Prouty and Gunn, (2000) demonstrated that *S.* typhimurium grown in the presence of up to 4% of bile could invade epithelial cells [44]. Furthermore, SP6 could have used multigrain efflux pumps to form a biofilm and therefore might be interrelated with the resistance system [45, 46].

Proposedly, the SP6 strain can come either from contaminated sediment, untreated manure, animal manure, and contaminated feeds attributed to sewage discharge, grazing animals along with rodents, reptiles surrounding the farm, and community disposal, including human and animal excreta [47], or during aquaculture with an unhygienic condition, capture, processing, storage, or preparation for consumption. Akiyama et al., (2011) suggested that *Salmonella* spp. isolated from shrimp can cause salmonellosis in humans because similar clinical isolates carry the same virulence gene [48]. *S. typhimurium* and *S. enteritidis* serovars cause most human salmonellosis related to shrimp consumption [10].

5. Conclusion

Based on our study, the recommendations would be the implementation of HACCP at the field level-farmers, suppliers, and depot owners by proper training. This will help minimize or eliminate various pollution sources. We also recommend washing fish with hyperchlorinated water that is considered as the crucial CCP. Chemical-free and cost-effective minimal processing needs to be developed to effectively eliminate pathogens and simultaneously produce a shelf-stable and fresher product. This study′s significance might be masked by working mainly with one pathogen. However, this report is the first from Bangladesh where appropriate identification of *Salmonella* spp. is shown along with key virulence properties and antibiotic resistance

Abbreviations

CCP:	Critical control point
HACCP:	Hazard analysis critical control point
HCl:	Hydrochloric acid
NaCl:	Sodium chloride
Salmonella spp:	*Salmonella* species
S. enteritidis:	*Salmonella enterica* serovar enteritidis
S. typhimurium:	*Salmonella enterica* serovar typhimurium.

Data Availability

The data that support the findings of this study are available in the supplementary material of this article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Prosanto Kumar Das, Ananta Mandal, and Md. Mizanur Rahman approved and read the manuscript. The authors contributed equally to this work.

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Supplementary Materials

Table S1: the Biochemical analysis results along with the PCR-based molecular assay for each presumptively identified bacteria. Table S2: the biofilm Ring test result at different salt concentrations. Table S3: antibiotic Susceptibility. Test Figure S1: invA gene-based PCR of *Salmonella* spp. (a) SP6 and S3g isolates were positive for the invA gene, (b) isolates B3B*D, B1C1F, G2B0D, G2B0E, B3B*D2, B3B*E, G3A1D, G3A1E, and G3A1F were positive for the invA gene; lane-ATCC for positive control. Figure S2: STM4497 gene-based PCR of *S.* typhimurium; (a) the SP6 isolate was positive for the STM4497 gene; (b) isolates G3A1F and G2B0D were positive for the STM4497 gene. Figure S3: STM3098 gene-based PCR of *S.* enteritidis; isolates G3A1D, G3A1E, and B3B*D were positive for the STM3098 gene. Figure S4: biofilm formation on glassware. The Salmonella typhimurium strain SP6 forms biofilms up to 4% NaCl whereas the *S.* enteritidis strain B-3B*D forms biofilms up to 8% salt concentration. (*Supplementary Materials*)

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