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Prevalence and PCR Detection of *Salmonella* spp. and *Escherichia coli* in Water Used during Live Transportation of Climbing Perch (*Anabas testudineus*) in Bangladesh

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Abstract. This research investigated the incidence of two foodborne pathogens, *Salmonella* spp. and *Escherichia coli* in water used during live transportation of climbing perch (*Anabas testudineus*). Experiments were conducted in three commercially important fish supply channels of Bangladesh, starting from an important production area, Muktagacha, Mymensingh to Dhaka (supply channel 1), Sylhet (supply channel 2), and Rajshahi (supply channel 3). Water samples were collected from 0 hours (at the time of loading) to reaching at the final destination (unloading points/ retail markets) at every 2 hours interval during transportation. To assess the prevalence of *Salmonella* spp. and *E. coli*, *Salmonella-Shigella* (SS) agar and Eosin methylene blue (EMB) agar plate counts were done. For confirmation and PCR detection, a total of 52 isolates were obtained from those cultured plates based on the colony characteristics. The finding showed a gradual increase in SS- and EMB agar plate counts in all the supply channels. Out of the 52 isolates, 10 (19%) were detected as positive for *Salmonella* spp., while 38 (73%) as positive for *E. coli* and the rest were unidentified. It was assessed that the pond waters of the cultured areas might be the primary sources of contamination of these two foodborne pathogens.

Keywords: Live transportation; Climbing perch; *Salmonella*; *Escherichia coli*; PCR detection.

1. Introduction

Transportation of fish in live condition is a common practice and the fish farmers' transport fish for various purposes and for variable periods of time (Stieglitz *et al*., 2012). Compared with fresh, chilled or frozen product, live aquatic organisms are regarded as products of higher commercial value as it maintains the better freshness and quality (Araujo *et al*., 2020). Live transportation of fishes before retailing, provide a substantially higher prices and reduce the processing costs (Reynisson *et al*., 2009). There are two conventional live fish transport strategy- namely open containers and closed containers delivery (Amend *et al*., 1982; Das *et al*., 2015). Similarly in Bangladesh, fish in live condition is usually transported either "open truck system" or "tank system" (Rajts *et al*., 2020). In case of the "open truck system", a plastic/ tarpaulin is usually placed on the truck bed to hold water and here the water volume varies with the carrying capacity of the truck. A certain percentage of live fish are transported in water-filled plastic containers where each container contains 35-40 kg of fish (Alam *et al*., 2010). The climbing perch (*Anabas testudineus*) locally known as 'Koi' is an important air-breathing freshwater species widely distributed in small rivers, canal and

swamp of Bangladesh (Mijkherjee *et al*., 2002). The species is considered as a valuable table fish for sick and convalescents, as it is a great source of protein with a high amount of iron and copper, which are mostly needed for hemoglobin synthesis (Sarma *et al*., 2010). In Bangladesh, commercial farming of this fish in the pond has become a very popular practice after the development of its induced breeding technique and mass production of seeds (Chhanda *et al*., 2019). Because of the consumers' preferences, this fish is commonly transported to the retail markets as live condition, especially to the major metropolitan areas. In Mymensingh area, the fishes are usually harvested using a large seine net, keep in "hapas" (net made rectangular barriers) setting at the corner of the fish ponds for acclamation usually for about 2 hours before transportation (Bhuiyan *et al.*, 2022; Hossain *et al*., 2021). For transportation, plastic barrel (1000 L) is loaded to commercial trucks with maximum carrying capacity of 5.17 tons. Plastic barrels are filled with 500 L of groundwater and each barrel was filled with 35 kg of fish approximately (on average 3 fishes per kg weight) and a maximum of 40 barrels are placed on each of the truck. No water additives, aeration facilities, and water exchange was practiced throughout the transportation (Hossain *et al*., 2021).

Bacteria are ubiquitous in the aquatic environment (Allen *et al*., 1983). Food from aquatic environments especially fish from aquaculture have been recognized as a major vehicle of foodborne pathogens to humans (Hradecka *et al*., 2008; IAEA, 2005). Human infections and intoxications caused by pathogens transmitted from fish are quite common and several species as *Salmonella* spp., *Staphylococcus* spp., *Vibrio* spp., *Aeromonas* spp., *Escherichia coli*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Staphylococcus aureus*, and *Listeria monocytogenes* has been isolated from fish responsible for serious health hazards to the fisherman, fish handlers and finally to the consumers (Novotny *et al*., 2004; Hassan *et al*., 1994). The two most prevalence enteric pathogens over the world are *Salmonella* spp. and *E. coli* where about 12% of the food poisoning outbreaks associated with fish consumption are caused by *Salmonella* spp. and according to the Brazilian surveillance service, after *Salmonella* spp. and *Staphylococcus aureus*, *E. coli* is the next etiological agent mostly liable for foodborne diseases (Da Silva *et al*., 2010; Carmo *et al*., 2005).

From aquaculture ponds *Salmonella* spp. and *E. coli* has been isolated in Bangladesh along with many other countries of the world (Ava *et al*., 2020; Haider *et al*., 2007). These two pathogens also indicate the status of biosecurity measures as well as the sanitary condition of the aquaculture ponds and subsequent supply channels. Based on all of these above facts and findings, here we hypothesized that although groundwater is used to transport the fishes, these two pathogens will be detected from the samples of the transport water as well as from the cultured pond waters. We also assumed that as transportation progress, their counts will gradually increase. So, the objective of this study was to verify these hypotheses. Three supply channels of live climbing perch transportation were selected and sub-samples were collected from the beginning (at 0h of loading) of the supply channel to the end/ final destination (at point of unloading).

2. Materials and Methods

2.1 Geographical Location of the Supply Channels

The study was carried out in three supply channels of climbing perch in Bangladesh. All the channels started from Muktagacha upazilla (24°44′01″N, 90°20′36″E) under Mymensingh division of Bangladesh to Showarighat, Dhaka

(23°42′25″N, 90°24′34″E), supply channel 1; to Poschim Kazir Bazar, Sylhet (24°88′80″N, 91°86′40″E), supply channel 2; and Shaheb Bazar, Rajshahi (24°21'53″N, 88°35'66″E), supply channel 3 (Fig. 1). The samplings were done from July to September, 2019.

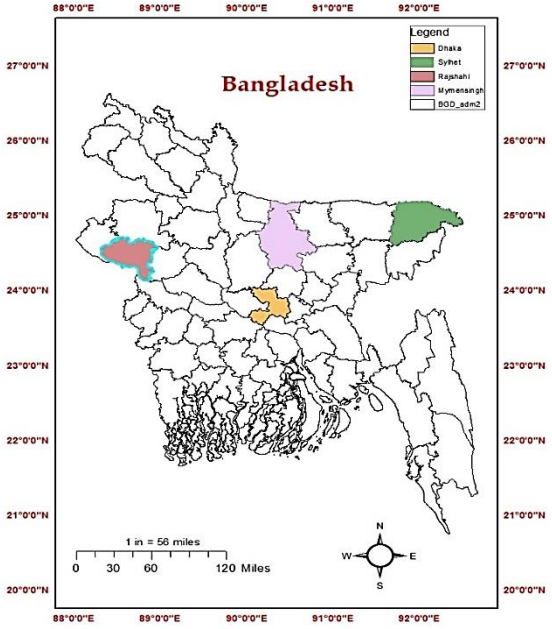

Fig. 1. Location of the studied supply channels of live climbing perch (*Anabas testudineus*) in Bangladesh. (Channel 1: Muktagacha, Mymensingh to Showarighat, Dhaka; Channel 2: Muktagacha, Mymensingh to Poschim Kazir Bazar, Sylhet; Channel 3: Muktagacha, Mymensingh to Saheb Bazar, Rajshahi). Geographical Information System (GIS) was used to extract images from DIVA-GIS. The development of map was done by ArcMap version 10.5.

2.2 Harvest, Post-harvest Handling of Fish for Transportation and Subsample Collection

Harvest of the fish was usually done at the late afternoon (from commercial climbing perch farms) for transportation at the following night. The details post-harvest preparation and procedures for transportation of climbing perch has been described in (Hossain *et al*., 2021). The fishes were captured using a large seine net and acclimated in a "hapa" (net made rectangular barriers measuring 3m × 2m × 1m) for about 2 hrs before loading. The fishes were transported in plastic barrels of 1000 L capacity loaded in a truck, which were filled with 500L of ground water and about 35 kg of fishes.

For sample collection, necessary sampling materials like beakers, volumetric flasks, tips, plastic containers, test tube, physiological saline, test tube, L-shaped glass rod, micropipette, hand gloves etc. were thoroughly washed and sterilized. SS- and EMB-agar plates were prepared in the Fisheries Microbiology Laboratory, Bangladesh Agricultural University by using SS- and EMB-agar media (Himedia, India). Prepared plates and other sampling materials/ tools were carried within a portable ice box (~4ºC) to the loading points. Travelling on the same vehicles used for the transportation of climbing perch was made to follow the changes in the counts and prevalence of *Salmonella* spp. and *E. coli* during transportation.

Subsamples of water were collected randomly in triplicate from the mid layer of the fish containing plastic barrels and kept in the previously sterilized plastic bottles (250 ml) started from 0h (at the time of loading fish to the truck) up to reach to the unloading points (desired fish retail markets) at every 2 hours interval. This approach allowed us to track the bacterial growth; decline or any fluctuations within the live fish transportation system/ transport water over time. During each sampling, water temperature was recorded using a glass thermometer and measured as °C. In order to assess the probable sources and prevalence of *Salmonella* spp. and *E. coli*, water samples were also collected from different cultured ponds of the sampling areas. The subsamples were divided into 3 groups and described under 3 collection sites, designated as collection point 1, 2, and 3. In this case, water samples were collected randomly in triplicate from different spots of the ponds. Subsequent preparation and plating was carried out onsite

immediately after collection of the water samples as described to the next step.

2.3 Sample Preparation, Culture on Agar Plates and Quantification

One ml of collected water sample was transferred to a test tube containing 9 ml of previously prepared and sterilized physiological saline (0.85% w/v NaCl) with a micropipette (Kang *et al*., 2018). The test tube was shaken thoroughly for mixing and 10-fold serial dilutions were prepared as 10^{-1}, 10^{-2}, 10^{-3}. Then 0.1 ml of the above dilutions were pipetted out and transferred aseptically to the previously prepared plate count agar plates (for bacterial viable counts) and SS- and EMB-agar plates (for SS- and EMB-agar plate counts) with replication of each dilution, and spreaded on the surface of the media using a L-shaped glass rod until the samples were dried out. All the cultured plates were then covered with aluminum fuel and kept in a specimen transport box until transferred to the Fisheries Microbiology Laboratory, Bangladesh Agricultural University, Mymensingh, Bangladesh. The plates were then incubated at 30°C for 48 hours (for bacterial viable counts) and at 37°C for 36 hours (for SS- and EMB-agar plate counts). Finally, bacterial viable counts, SS- and EMB-agar plate counts were calculated by using the following formula-

cfu/ml = no. of colonies on petridish × 10 × dilution factor.

2.4 Isolation and Identification of Salmonella spp. and Escherichia coli

The isolation and identification of *Salmonella* spp. and *E. coli* was carried out based on culture on the SS- and EMB- agar plates described in (Haider *et al*., 2007) with slight modification. For this purpose, bacterial colonies developed on SS- and EMB-agar plates were collected (Fig. 2). Based on the colony characteristics, 3-5 representative colonies of each type were then purposively

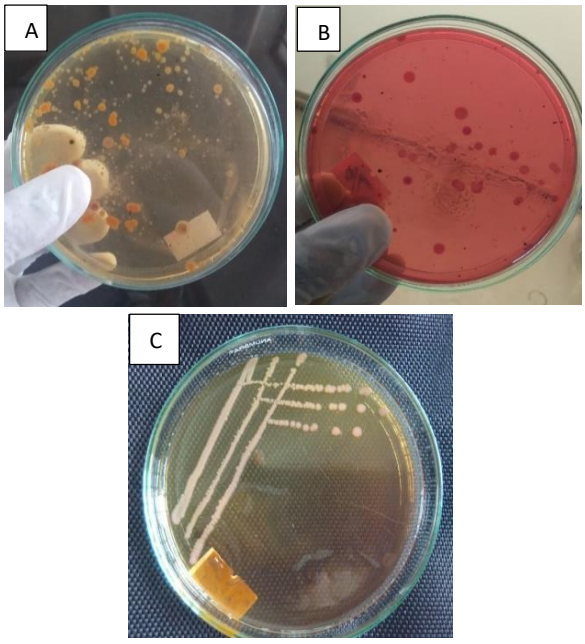

Fig. 2. Culture and growth of bacteria onto selected agar media conducted to detect *Salmonella* spp. and *Escherichia coli* in water used during live transportation of climbing perch (*Anabas testudineus*) in Bangladesh; A) suspected colonies of *Salmonella* spp. onto SS-agar medium, B) growth of *Escherichia coli* onto EMB-agar medium, and C) well-separated colonies of the targeted bacteria obtained after streaking onto SS-agar medium.

streaked on SS-agar (Himedia, India). After that plates were incubated aerobically at 37°C for 24 hours and well isolated colonies were observed. Repeatedly streaking was performed to get well separated colonies wherever necessary (Fig. 2). On the SS-agar plates, colonies with black-center, having opaque color or colorless characteristics were presumptively considered as indicative of *Salmonella* spp., and colonies with cream or pink to red color were presumptively considered as indicative of *E. coli*. The final confirmation of the isolation of *Salmonella* spp. and *E. coli* was performed by polymerase chain reaction (PCR) targeting the specific invA gene of *Salmonella* spp. and partial 16S rRNA gene of *E. coli*.

2.5 Extraction and Amplification of Bacterial DNA

For PCR, genomic DNA was extracted from *Salmonella* spp. and *E. coli* suspected pure cultures (isolates) by the boiling method described by (Rawool *et al*., 2007). In brief, a pure colony was put into an Eppendorf tube containing 100 µL of deionized water and gently vortex, followed by boiling and cooling for 10 min during each step. Finally, genomic DNA was collected after centrifugation for 10 min and stored at -20°C for further use.

Detection of *Salmonella* spp. invasive encoding gene and partial 16S rRNA gene of *E. coli* was done by using thermal cycler (ABI 2720, Applied Biosystems, Singapore). PCR primers and conditions used in the current study are provided in (Table 1) with the expected product size. Total PCR reaction mixture was 25 µl, having 12.5 µl 2× Master Mix (Promega, Madison, WI, USA), 1.0 µl of forward primer (10 pmol/µl), 1.0 µl of reverse primer (10 pmol/µl), 5.0 µl of DNA template, and 5.5 µl of nuclease-free water. After completion, the amplified PCR products were analyzed by gel electrophoresis (Cleaver Scientific, UK) using 1.5% agarose gel in 50× Tris-Acetic acid EDTA

Table 1. Primers used for the detection of *Salmonella* spp. and *Escherichia coli* from the water used during live transportation of climbing perch (*Anabas testudineus*) at different supply channels of Bangladesh and PCR conditions.

Bacteria	Target Gene	Name of Primer	Primer sequence (5`-3`)	PCR conditions	Amplicon size (bp)	References
Salmonella spp.	*invA*	InvA F	ATCAGTACCAGTCGTCTTATCTTGAT	Initial denaturation: 94°C for 5 min Denaturation: 94°C for 30 sec Annealing: 52°C for 2 min Extension: 72°C for 45 sec Final extension: 72°C for 5 min Cycles: 30	211	(Ogunremi *et al*., 2017)
		InvA R	TCTGTTTACCGGGCATACCAT			
E. coli	16S rRNA	EC-1	GACCTCGGTTTAGTTCACAGA	Initial denaturation: 94°C for 5 min Denaturation: 94°C for 45 sec Annealing: 52°C for 45 sec Extension: 72°C for 1 min Final extension: 72°C for 5 min Cycles: 30	585	(Hassan *et al.*, 2014)
		EC-2	CACAGCTGACGCTGACCA			

(TAE) buffer. Amplicons were stained by ethidium bromide (0.5 μg/ml) and visualized under an ultraviolet trans-illuminator (Biometra, Germany). A 100 bp DNA ladder (Promega, Madison, WI, USA) was used as a molecular weight marker of the PCR amplicons.

2.6 Statistical Analyses

All the generated data and the results of laboratory investigations were entered, edited, coded, and analyzed using Microsoft Office Excel (2013). The quantitative values of bacteria were obtained using the formulae mentioned before. The error bars in the graphs were obtained by determining the standard deviations (SDs) of the triplicated values using Microsoft Office Excel (2013). A one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test was conducted to evaluate the significant difference in viable bacterial count, SS, and EMB agar plate counts among the different sampling channels at 5% ($p<0.05$) level of significance through SPSS (version 26).

3. Results

3.1 Length of the Supply Channels and Changes in Temperature of the Transport Waters

The distances between the loading points to the unloading points were about 140 km, 305 km, and 230 km, respectively for the supply channel 1, channel 2, and channel 3. It took about 6 hrs, 8 hrs, and 8 hrs to reach the destination in the case of supply channel 1, 2, and 3, respectively due to variations in traffic conditions along the route.

The temperature of the transport waters were quite similar among the supply channels and varied between 29-31 °C regardless of the sampling periods.

3.2 Changes in Bacterial Viable Counts, SS- and EMB-ager Plate Counts in Water Used during Live Transportation

Bacterial viable counts demonstrated an upward trend with the duration of transportation. In channel 1, within 6 hours of transportation, the counts increased from

$(0.64 \pm 0.17)\times10^4$ cfu/ml to

$(31.01 \pm 11.55)\times10^4$ cfu/ml

While after 8 hours of transportation in channels 2 and 3, it increased from $(0.56 \pm 0.16)\times10^4$ cfu/ml to $(47.52 \pm 19.23)\times10^4$ cfu/ml and $(0.69 \pm 0.04)\times10^4$ cfu/ml to $(41.01 \pm 17.42)\times10^4$ cfu/ml, respectively. However, statistical analysis revealed no significant difference ($p > 0.05$) in viable bacterial counts among the studied supply channels at the same period of transportation (Fig. 3 and supplementary Table S1).

The average SS-agar plate counts were $(0.13 \pm 0.01)\times10^4$, $(0.21 \pm 0.03)\times10^4$, and $(0.12 \pm 0.05)\times10^4$ cfu/ml at the beginning (at 0 h) in case of supply channel 1, channel 2, and channel 3, respectively. Regardless of the supply channels, a gradual increase in SS-agar plate counts were observed in all the cases that were $(1.06 \pm 0.06)\times10^4$, $(1.37 \pm 0.07)\times10^4$, and $(1.28 \pm 0.11)\times10^4$ cfu/ml, respectively in channel 1, channel 2, and channel 3 at the final destination before the periods of unloading (6h, 8h, and 8h, respectively in case of channel 1, channel 2, and channel 3). Although bacterial counts on SS agar varied significantly ($p < 0.05$) among the supply channels at 0h, 2h, and 4h but it was insignificant ($p > 0.05$) at 6h, and 8h of transportation (Fig. 3 and supplementary Table S2). Similar tendencies, *i.e*. gradual increase in EMB- agar plate counts were also observed in all of the studied supply channels. The initial averages of EMB- agar plate counts were $(0.23 \pm 0.04)\times10^4$ cfu/ml, $(0.22 \pm 0.05)\times10^4$ cfu/ml, and $(0.13 \pm 0.03)\times10^4$ cfu/ml, respectively in channel 1, channel 2, and channel 3 which were finally increased to:

$(1.49 \pm 0.08)\times10^4$ cfu/ml, $(1.8 \pm 0.09)\times10^4$ cfu/ml, and $(1.45 \pm 0.05)\times10^4$ cfu/ml, respectively. However, a significant variation ($p < 0.05$) of EMB agar plates count were observed among the studied supply channels at 2 h, 4 h, 6 h, and 8h of transportation (Fig. 3 and supplementary Table S3).

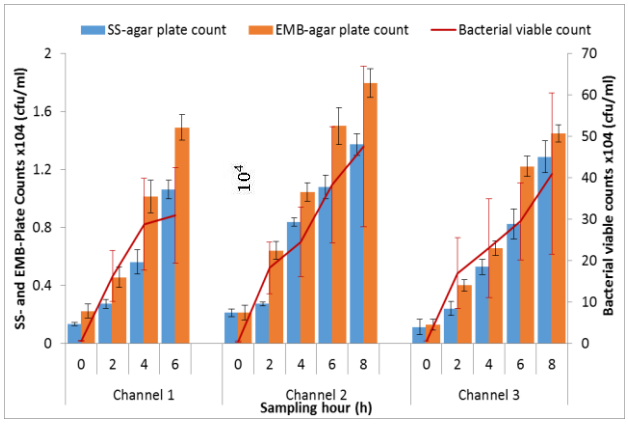

Fig. 3. Comparison in changes of SS- and EMB-agar plate counts (columns) with bacterial viable counts (lines) of water used during live transportation of climbing perch (*Anabas testudineus*) at different supply channels of Bangladesh (cfu = colony forming unit). The error bars are indicating the standard deviations (SDs) of the triplicated values.

3.3 PCR Detection of Salmonella spp. and E. coli in Water Used during Live Transportation

In the present study, for PCR detection of *Salmonella* spp. and *E. coli* from water used during live transportation of climbing perch was done after conventional culture based screening of isolates. A total number of 52 isolates were collected from all the three supply channels. Two sets of primers such as, InvA (211 bp) targeting invA genes of *Salmonella* spp., and EC (585 bp) targeting parts of 16s rRNA genes of *E. coli* were used during PCR. In case of sampling channel 1, out of 14 isolates, 2 (14.28%) isolate were detected as positive for *Salmonella* spp. (Fig. 4A), while 10 (71.42%), isolates as positive for *E. coli* (Fig. 4B). In case of sampling channel 2, out of 21 isolates, 5 (23.80%) were detected as positive for *Salmonella* spp. (Fig. 4C), while 15 (71.43%) as positive for *E. coli* (Fig. 4D). And finally in case of supply channel 3, 17 isolates were obtained of which 3 (17.64%) were found positive for *Salmonella* spp. (Fig. 4E), and 13 (76.47%) for *E. coli* (Fig. 4F). Overall, out of 52 isolates, 10 (19%) isolates were detected as positive for *Salmonella* spp., while 38 (73%) for *E. coli*, and 4 (8%) isolates were not provided any band after PCR, recognized as unidentified (Table 2 and Fig. 5).

3.4 SS- and EMB-Ager Plate Counts of the Cultured Ponds of the Studied Areas

In order to assess the SS- and EMB- agar plate counts of the climbing perch cultured ponds, water samples were collected from 13 different ponds of three particular collection points. Following established procedures (Haider *et al*., 2007) the water samples were plated, and incubated at 37°C for 36 hours. In case of collection point 1, 4 sampling ponds were assessed in which the average SS-agar plate counts were about $(0.35 \pm 0.08)\times10^4$ cfu/ml, $(0.43 \pm 0.07)\times10^4$ cfu/ml, $(0.41 \pm 0.10)\times10^4$ cfu/ml, $(0.45 \pm 0.14)\times10^4$ cfu/ml, respectively, while average EMB-agar plate counts were $(0.64 \pm 0.06)\times10^4$ cfu/ml, $(0.77 \pm 0.08)\times10^4$, $(0.49 \pm 0.07)\times10^4$, $(0.78 \pm 0.12)\times10^4$ cfu/ml respectively. Furthermore, at collection points 2 and 3, water samples were collected from 5 and 4 cultured ponds, respectively. The average SS- and EMB-agar plate counts for collection point 2 were $(0.45 \pm 0.07)\times10^4$ cfu/ml, and $(0.69 \pm 0.06)\times10^4$ cfu/ml, respectively. However, at the collection point 3, the counts slightly varied with $(0.41 \pm 0.10)\times10^4$ cfu/ml for SS-agar plates and $(0.72\pm 0.12)\times10^4$ cfu/ml for EMB-agar plates (Fig. 6). The results showed that all the pond water gave counts on SS- and EMB-agar plates and the counts of the pond waters were somewhat close to the initial (0 h) counts of the supply channels (Fig. 3 and 6).

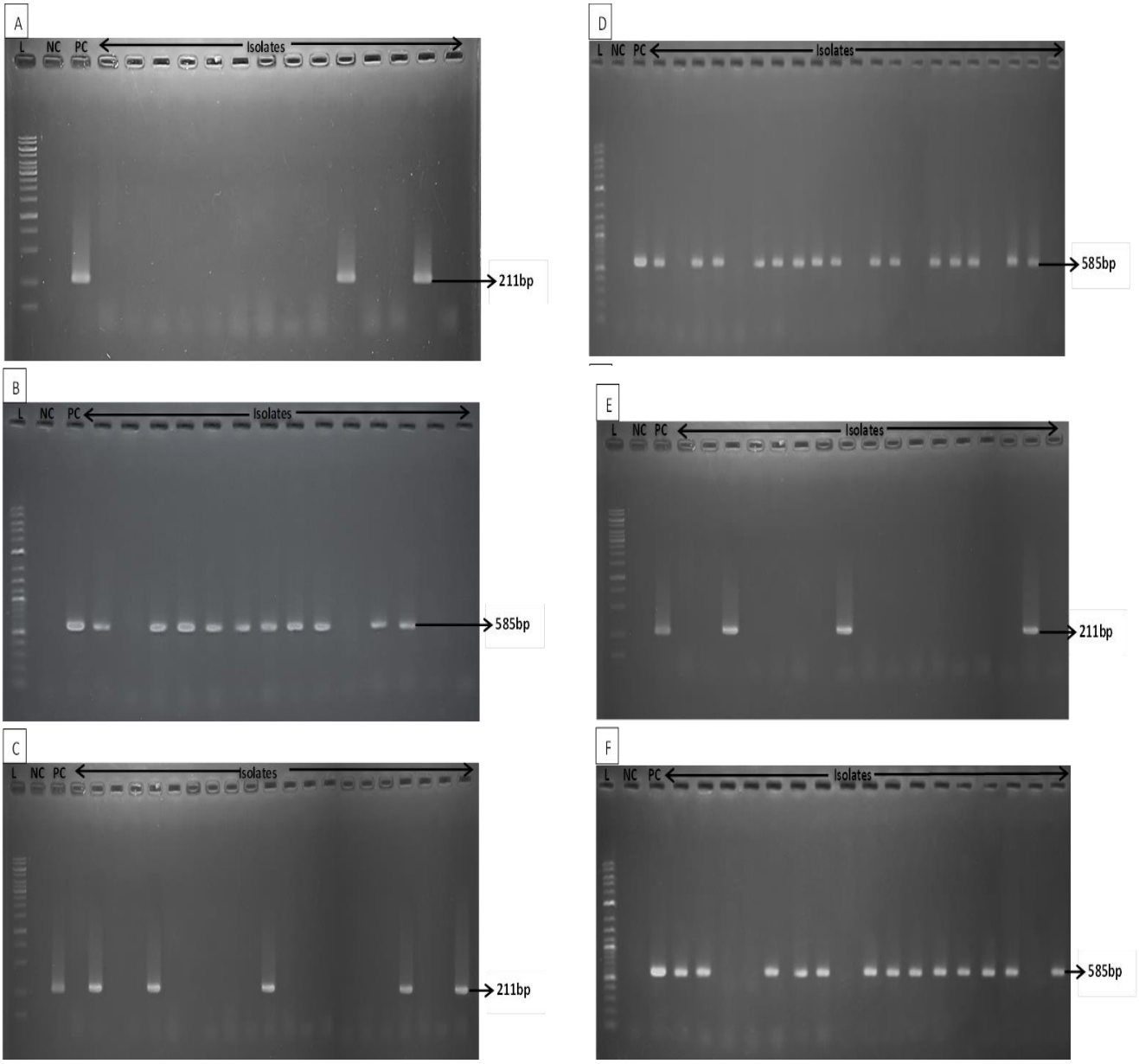

Fig. 4. Amplification of *invA* gene of *Salmonella* spp. (211 bp) and partial 16S rRNA gene of *E. coli* (585 bp) isolates recovered from different supply channels of climbing perch (*Anabas testudineus*); A) *Salmonella* spp. isolates from supply channel 1, B) *E. coli* isolates from supply channel 1, C) *Salmonella* spp. isolates from supply channel 2, D) *E. coli* isolates from supply channel 2, E) *Salmonella* spp. isolates from supply channel 3, and F) *E. coli* isolates from supply channel 3. In the lanes, L = ladder, NC = negative control, and PC = positive control.

Table 2. Summary of the obtained isolates, PCR analyses and detection of *Salmonella* spp. and *Escherichia coli* from the water used during live transportation of climbing perch (*Anabas testudineus*) at different supply channels of Bangladesh.

Supply channel	Time required to final destination (h)	No. of collected isolates	No. of isolates tested positive for *Salmonella* spp.	No. of isolates tested positive for *E. coli*	No. of identified isolates	No. of unidentified isolates	Incidence of *Salmonella* spp. (%)*	Incidence of *E. coli* (%)*	Percentage (%) of unidentified isolates*
1	6	N=14	2	10	12	2	14.28	71.42	14.28
2	8	N=21	5	15	20	1	23.80	71.43	4.76
3	8	N=17	3	13	16	1	17.64	76.48	5.88

Note. N=Total number of isolates obtained, * % = (No. of isolates found positive or unidentified /N) ×100

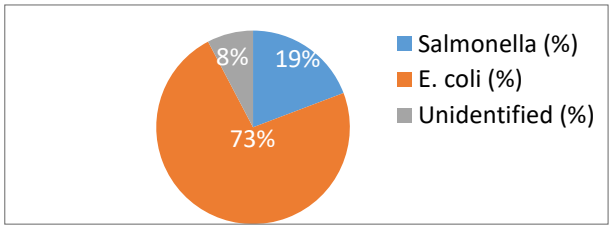

Fig. 5. Percentage of *Salmonella* spp. and *E. coli* positive isolates obtained from the water used during live transportation of climbing perch (*Anabas testudineus*) at different supply channels of Bangladesh; % unidentified indicating those did not give any bands for the used primer sets.

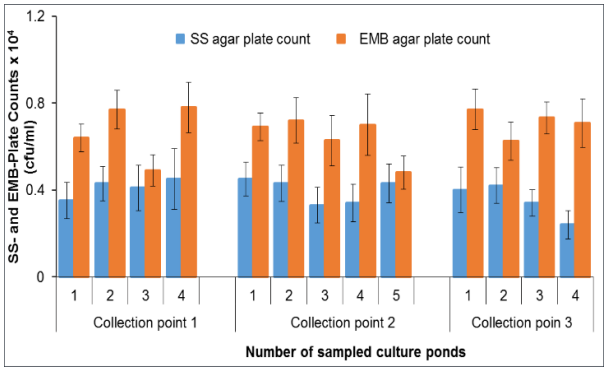

Fig. 6. SS- and EMB-agar plate counts of the water collected from the climbing perch (*Anabas testudineus*) cultured ponds of three different collection points in the studied areas of Bangladesh. The error bars are indicating the standard deviations (SDs) of the triplicated values.

4. Discussion

We hypothesized that these two pathogens *Salmonella* spp. and *Escherichisa coli* will be detected from the water samples used during live transportation of fish and cultured ponds, and their counts will gradually be increased in water used during live transportation of fish. The detection of *Salmonella* spp. and *E. coli* from live climbing perch transport water is a great concern due to its potential to cause enteric diseases, which are globally recognized as foodborne zoonoses (Oh *et al*., 2011).

Both culture based (in selective media) and molecular based approaches were used for identification and confirmation of these two bacteria. Several studies have been applying for the isolation and identification of *Salmonella* and *E. coli* from the food samples. For culture based identification several selective media are used. *Salmonella* spp. produce opaque, translucent and colorless colonies on *Salmonella-Shigella* (SS) selective agar while colorless, pale, transparent colonies on MacConkey agar (Rather *et al*., 2013). *E. coli* on the other hand, appear as pink colonies (lactose fermentation) on MacConkey's agar medium, whereas, a characteristic greenish metallic sheen on EMB agar medium (Shahzad *et al*., 2013; Abd El-Aziz *et al*., 2014). PCR

based molecular methods for detection of *Salmonella* spp. and *E. coli* was performed in this study. However, PCR detection provides more confirmatory results in a short time with high accuracy. It is important to note that primers used in this study for detection of *Salmonella* spp. and *E. coli* are known as very fast, unique, sensitive in detecting by PCR examination technique (Yanestria *et al*., 2019). For this reason, polymerization of invA gene has been popularly using for the detection of *Salmonella* from different types of samples (Malorny *et al*., 2003; Van Blerk *et al*., 2011; Aliviameita *et al*., 2011; Ogunremi *et al*., 2017; Yanestria *et al*., 2019). Seel *et al*., (2016) conducted a study on identification of *Salmonella* spp. from *Anabas testudineus* through molecular detection targeting the invA gene. While for *E. coli*, amplifying genus specific 16S rRNA gene by PCR using EC-585bp primer give confirmatory results (Maheux *et al*., 2009; Uddin *et al*., 2019).

Several pathogenic genera of bacteria (either for fish or human being) such as *Aeromonas* spp., *Vibrio* spp., *Salmonella* spp., and *E. coli* has been isolated from the commercial climbing perch farming areas (Zaman *et al*., 2013). However, there is scarcity of information on the prevalence of *Salmonella* spp., and *E. coli* in water used during live transportation of fish in Bangladesh as well as in the world. Also, there is no data about their quantitative changes during live transportation of fishes in Bangladesh. This study is the first attempt in Bangladesh has made to assess the prevalence and PCR based detection of *Salmonella* spp. and *E. coli* in water used during live transportation of climbing perch. Previously, it was reported (Hossain *et al*., 2021) that regrowth of bacteria is occurred and the viable counts gradually increase in the water used during live transportation of climbing perch.

In aquaculture, the health of fish is mostly influenced by the water conditions of a fish farm (Roalkvam *et al*., 2019). Fish is known to harbour bacteria of public health importance. Bacterial loads especially enteric bacterial population of the harvested fish reflects the microbial water quality of the pond. The supplying of live fish is routinely delivered by small volume of water. The results of the study showed that the water of climbing perch cultured ponds gave enteric bacterial counts on SS- and EMB-agar plates. This is may be because of introduction of or contamination by animal excreta/ wastes to the fish producing ponds or fecal contamination of the pond water from the areas (Chowdhury, 2010). Sewage contamination or waste water contamination from human toilets are reported to contain bacterial and other parasitic pathogens (Kay *et al*., 2008) are also contaminate the pond water during heavy rain and surface runoff. Moreover, several authors (Ava *et al*., 2020; Chhanda *et al*., 2019; Hossain *et al*., 2017; Zaman *et al*., 2013) detected *Salmonella* spp. and *E. coli* from climbing perch cultured pond waters.

Salmonella spp. and *E. coli* found in the intestianl tracts of animals and can shed in the faeces which can contaminate food, soil and water. *Salmonella* spp. can also survive for months in the soil. Previous reports also showed that *Salmonella* spp. can survive in the environment for several years (Gorski *et al*., 2011). Although, it cannot multiply in water because of sufficeient nutrients, it was found that in the groundwater they may remain as viable state (Pronk *et al*., 2006). *Salmonella* spp. and *E. coli* can introduce to the fishes from the contaminated waters. After contamination, fish itself may become host of *Salmonella* with no clinical signs (Bibi *et al*., 2015). Previous reports also showed a high prevalence of *Salmonella* spp. in fish intestines, skin, gills (Nwiyi and Onyeabor, 2012) and fish muscles (El-Olemy *et al*., 2014). In addition to the *Salmonella* spp. and *E. coli*, there are so many other enteric bacteria, including pathogenic

species are naturally present in the freshwater, capable of growing there (Hendricks, 1972; Hendricks and Morrison, 1967). Accumulation of fecal materials and metabolic wastes within the waters can also enhance the growth of bacteria during climbing perch transportation (Hossain *et al*., 2021).

When fish are consistently exposed to the microorganisms present in the water bodies and fish itself is a source of bacteria (Mayer and Ward, 1991). For this reason, although groundwater is generally used for live transportation they may be contaminated from the fish itself. Morover, groundwater can also be contaminated by a wide range of pathogens including enteric groups as reported before (Li *et al*., 2009; John and Rose, 2005). Pathogenic bacteria such as *Salmonella* spp, *Shigella* spp, *Vibrio cholerae* and *E. coli* being shed in human and animal faeces ultimately find their way into water supply through seepage of improperly treated sewage into groundwater (Dipaola, 1998). Three important members of the family Enterobacteriaceae *i.e. E. coli, E. aerogenes*, *Salmonella* and *Klebsiella* had identified in groundwater by (Suthar *et al*., 2009). Even in developed countries, contamination of groundwater by microbial pathogens had also been documented (Borchardt *et al.*, 2004; Bockelmann *et al.*, 2009). Another possible source of these pathogenic bacteria can be the transport containers used during live transportation. In Bangladesh, fish transporters used plastic barrels that are often uncleaned and being used frequently without washing and cleaning after transporting a batch. Even Microorganisms can survive and thrive in the sand of fish-holding plastic water tanks and (Momba and Kaleni, 2002) isolated *Escherichia coli*, *Salmonella* and *Clostridium perfringens* from plastic water containers.

So, the gradual increase of the SS- and EMB- agar plate counts of the water used during live transportation of climbing perch may be because of longer exposure of the fishes to the limited amount of water in the transporting tanks as well as excretory activities for longer period of time. Anyway, it should be noted here that SS- and EMB-agar support the growth of some other enteric bacteria other than *Salmonella* spp. and *E. coli*, thus the SS- and EMB-agar plate counts are not necessarily indicating the growth of only *Salmonella* spp. and *E. coli*. However, further intensive investigation regarding the growth and multiplication of enteric bacteria in the presence of a higher amount of excretory materials (also nutrients for some bacteria) within the system should be taken into consideration for further clarification of the reasons of gradual increase of SS- and EMB-agar plate counts.

5. Conclusion

The findings of this study indicate that the water used during live transportation of climbing perch contained bacteria of enteric origins. The PCR detection of the isolates showed that pathogenic groups like *Salmonella* spp. and *E. coli* are also included to this enteric group. This is the first report on the prevalence of *salmonella* spp. and *E. coli* in water used during live transportation of fish in Bangladesh. For the sake of consumers' safety issues, the rules of the good aquaculture practices and good handling practices should be taken into consideration.

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Supplementary Data

Table S1. Variability in viable bacterial counts of water used during live transportation of climbing perch (*Anabas testudineus*) at different supply channels of Bangladesh (cfu = colony forming unit).

Time (h)	Bacterial viable counts (mean ±sd)× 10^4 cfu/ml		
	Channel 1	**Channel 2**	**Channel 3**
0	0.64 ± 0.17[a]	0.56 ± 0.16[a]	0.69 ± 0.04[a]
2	16.32 ± 6.10[a]	18.27 ± 6.16[a]	16.98 ± 8.53[a]
4	28.85 ± 11.08[a]	24.55 ± 8.42[a]	23.07 ± 11.90[a]
6	31.01 ± 11.55[a]	38.52 ± 13.99[a]	29.52 ± 9.25[a]
8	-	47.52 ± 19.23[a]	41.01 ± 17.42[a]

Values are expressed as mean ±SD. Means in the same row (counts at the same period of transportation) with a same superscript are not significantly (P>0.05) different.

Table S2. Variability in SS- agar plate counts of water used during live transportation of climbing perch (*Anabas testudineus*) at different supply channels of Bangladesh (cfu = colony forming unit).

Time (h)	Bacterial counts SS agar (mean ±sd)× 10^4 cfu/ml		
	Channel 1	**Channel 2**	**Channel 3**
0	0.13 ± 0.01[b]	0.21 ± 0.03[a]	0.12 ± 0.05[b]
2	0.24 ±5.686[b]	0.36±2.00[a]	0.23 ±3.215[b]
4	0.69±16.166[ab]	0.86 ±4.163[a]	0.58 ±2.082[b]
6	1.06 ± 0.06[a]	1.09±2.517[a]	1.12 ±3.00[a]
8	-	1.37 ± 0.07[a]	1.28 ± 0.11[a]

Values are expressed as mean ± SD. Means in the same row (counts at the same period of transportation) with different alphabetical superscripts are significantly (p < 0.05) different.

Table S3. Variability in EMB- agar plate counts of water used during live transportation of climbing perch (*Anabas testudineus*) at different supply channels of Bangladesh (cfu = colony forming unit).

Time (h)	Bacterial counts on EMB agar (mean ±sd)× 10^4 cfu/ml		
	Channel 1	**Channel 2**	**Channel 3**
0	0.23±1.528[a]	0.22±1.041[a]	0.13±1.163[a]
2	0.48±4.041[a]	0.61 ±1.528[a]	0.34 ±2.646[b]
4	1.09 ±4.041[a]	1.02±2.646[a]	0.65 ±6.245[b]
6	1.49±3.00[a]	1.37±4.509[a]	1.13±7.00[b]
8	-	1.83±7.024[a]	1.45±6.110[b]

Values are expressed as mean ± SD. Means in the same row (counts at the same period of transportation) with different alphabetical superscripts are significantly (p < 0.05) different.

انتشار وكشف PCR للسالمونيلا والإشريكية القولونية في المياه المستخدمة أثناء النقل الحي لسمك الفرخ المتسلق (*Anabas testudineus*) في بنغلاديش

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المستخلص. التحقيق في هذا البحث في حدوث اثنين من مسببات الأمراض المنقولة بالغذاء، السالمونيلا. والإشريكية القولونية في المياه المستخدمة أثناء النقل الحي لسمك الفرخ المتسلق (*Anabas testudineus*). أجريت التجارب في ثلاث قنوات إمداد سمكية مهمة تجاريًا في بنغلاديش، بدءًا من منطقة إنتاج مهمة، موكتاجاتشا، وميمينسينغ إلى دكا (قناة الإمداد ١)، وسيلهيت (قناة الإمداد ٢)، وراجشاهي (قناة الإمداد ٣). تم جمع عينات المياه من الساعة صفر (وقت التحميل) حتى الوصول إلى الوجهة النهائية (نقاط التفريغ/أسواق البيع بالتجزئة) كل ساعتين أثناء النقل. لتقييم مدى انتشار السالمونيلا النيابة. وتم إجراء تعداد أجار الإشريكية القولونية وأجار السالمونيلا-الشيغيلا (SS) وأجار أجار الميثيلين الأزرق (EMB) للتأكيد والكشف عن تفاعل البوليميراز المتسلسل، تم الحصول على إجمالي ٥٢ عزلة من تلك الصفائح المزروعة بناءً على خصائص المستعمرة. أظهرت النتائج زيادة تدريجية في عدد ألواح أجار-SS وEMB في جميع قنوات الإمداد. من بين ٥٢ عزلة، تم اكتشاف ١٠ (١٩٪) إيجابية لبكتيريا السالمونيلا، بينما ٣٨ (٧٣٪) إيجابية لبكتيريا الإشريكية القولونية والباقي لم يتم التعرف عليهم. وقد تم التقييم أن مياه البرك في المناطق المستزرعة قد تكون المصدر الرئيسي لتلوث هذين المسببين للأمراض المنقولة بالغذاء.

الكلمات المفتاحية: النقل الحي؛ تسلق الفرخ السالمونيلا. الإشريكية القولونية؛ كشف PCR.