Indian Journal of Microbiology Research 2019;6(4):355–366

Content available at: iponlinejournal.com

Indian Journal of Microbiology Research

Journal homepage: www.innovativepublication.com

Original Research Article

Profiling resistance pattern and assessing toxicity of coliform bacteria on the developmental stages of *Artemia salina* isolated from drinking water of northern part of Bangladesh

Afrin Priya Talukder[1], Md. Nazmul Haque[1], Rashed Zaman[1], Md. Akhtar-E- Ekram[1,*]

[1]*Dept. of Genetic Engineering & Biotechnology, University of Rajshahi, Rajshahi, Bangladesh*

ARTICLE INFO

Article history:
Received 11-09-2019
Accepted 09-11-2019
Available online 21-11-2019

Keywords:
Coliform
Multi drug resistant (MDR)
Artemia salina
DMRT analysis
tD50
LC50

ABSTRACT

Objectives: Scarcity of safe drinking water is a major health concern in Bangladesh. In this context, both commercially and locally available drinking water in the northern part of Bangladesh were assessed.
Materials and Methods: Coliform bacteria were isolated on MacConkey agar media and morphological, physiological, biochemical, molecular characterization were also done. Antibiotic resistance pattern was tested through disc diffusion method. Acute toxicity assay was performed on stationary and exponential stages of *Artemia salina* development as well as significant variation was analyzed by Duncan multiple range test (DMRT) using statistical analysis software (SAS, version 9.1.3). Additionally, LC_{50} value was evaluated through probit mortality software against *Artemia salina*.
Results: Among twelve samples, on MacConkey agar screening illustrated highest number of coliform colonies in Sample 1 (Isolate A)> Sample 7 (Isolate B)> Sample 9 (Isolate C) and were selected on the basis of colony number and morphology for microbiological and toxicological profiling. Molecular identification using 16S rRNA gene sequencing showed isolate A, B and C as *Aeromonas* sp. with 76%, *Enterobacter* sp. and *Escherichia* sp. with 96% homogeneity, respectively. *Aeromonas* sp. and *Escherichia* sp. were multi-drug resistant against penicillin, ceftazidime, doxycycline and cefuroxime. Among three isolates, *Escherichia* sp. showed highest toxicity on both stages of *Artemia salina* with abnormal organ formation (atypical head width, swimming leg missing, deformed ovary) and no *Artemia salina* survival was found in exponential phase after 8 hour. tD_{50} values were 6, 7 and 4 hour for *Aeromonas* sp., *Enterobacter* sp. and *Escherichia* sp., respectively. Moreover, highest toxic LC_{50} value was 48.32 $\pm$0.17 μl for *Escherichia* sp. among three isolates.
Conclusion: The present study is the first considerable evidence of coliform bacterial toxicity on *Artemia salina* development which indicates detrimental consequences of consuming contaminated drinking water.

1. Introduction

Water is vital to life, nevertheless many people do not have access to clean and safe drinking water and many die of waterborne bacterial infections. Several diseases can be evoked by pathogenic microorganisms found in contaminated water.[1] In this context, water resources are the main stream carter of water borne pathogens which finally have adverse impact on human health and also on socio-economic environment. Most importantly, these pathogens are diverse in nature and placing entire communities at jeopardy.[2] Water quality assessing depends on the identification of disease producing microorganisms present in water and the best approach is the use of an easily measured "indicator organism" to signal that pathogenic microorganisms may be present and the coliform group of bacteria is the marker used worldwide.[3] Coliform bacteria include a large group of many types of bacteria (Coliform are defined as aerobic and facultative anaerobic bacteria, gram-negative, nonspore-forming and rod-shaped bacteria

* *Corresponding author*.
E-mail address: ekram_2012@ru.ac.bd (M. A. E. Ekram).

https://doi.org/10.18231/j.ijmr.2019.075

that ferment lactose with gas and acid formation within 48 hour when incubated at 37°C).[4] Most coliforms are present in large numbers among the intestinal flora of humans and other warm blooded animals and are therefore found in fecal wastes[5] and fecally contaminated drinking water is a main public health setback and cause of water transmit diseases.[6]

In developing countries, waterborne diseases are the major matter of concern regarding public health.[7] Worldwide, the burden of diarrheal disease is highest in Southeast Asia and Africa[8] and unsafe drinking water is the main cause of 1.8 million deaths of children aged below 5 years due to diarrheal diseases yearly.[9] If coliform bacteria are present in drinking water, risk of contracting a waterborne illness is raised. The main bacterial diseases transmitted through drinking water include Cholera, Typhoid fever, Bacillary dysentery or Shigellosis, Acute diarrheas, Gastroenteritis and other serious Salmonellosis.[10] Outbreaks of disease attributable to drinking water can escort to serious acute, chronic, or sometimes fatal health consequences.

In this perspective, Bangladesh is in a great peril as a developing country with uppermost density of population in the world. The percentage of utilizing ground water for drinking and household use is about 97% of the population of the country.[11] Scarcity of safe drinking water is a common problem for both the urban and rural areas in Bangladesh.[12] The mainstream (64%) of the urban population and almost all (93%) of the rural population have access to hand-pumped or piped water due to bacteriological contamination of surface water in Bangladesh.[13] Several studies have confirmed that surface water sources in Bangladesh are profoundly contaminated with fecal coliforms (FC) and by various pathogenic bacteria.[14] Most notably, environmental enteric dysfunction is an abnormality of gut function that might explain why most nutrition interventions fail to normalize early childhood growth.[15] It is reported that improvements to drinking water quality, sanitation, and hand washing might develop the effectiveness of nutrition interventions and thereby help to deal with a larger portion of the observed growth deficit.[16]

In recent years, bottled water consumption has increased significantly in Bangladesh. Presence of fecal indicator and heterotrophic bacteria have been reported with levels exceeding drinking water guidelines by various investigations.[17] Local newspapers and social media in Bangladesh have expressed their deep concerns that some brands of bottled water may be unsafe for consumption.[18] Pathogenic bacteria such as *Aeromonas sp.*,[19] *Pseudomonas sp.*,[20] *Shigella sp.*,[21] *Salmonella sp.*,[22] *Vibrio cholera*[23] etc have been perceived in bottled water. European Community Directive (European Community 1980) reported that total coliforms, *E.coli*, *Enterococcus* sp., *Pseudomonas aeruginosa* and parasites should not be identified in 250 ml of bottled water and World Health Organization[24] suggests that the number of fecal coliforms should be zero in drinking water. Hence, the present study was planned on the isolation, characterization, profiling resistance pattern of coliform bacteria from drinking water which acts as an "indicator organism" of other pathogenic bacteria and assessing their toxicity on the developmental stages of aquatic organism *Artemia salina* and so on.

2. Materials and Methods

2.1. Sample collection

Total twelve drinking water samples were collected during March, 2017. Among twelve samples, four were local drinking water samples (Sample1- Sample 4) which were collected from different areas of Rajshahi University campus and eight were bottled water samples (Sample 5-Sample 12) which were collected from different regions of Rajshahi. Samples were aseptically collected and brought to the Microbiology Laboratory, Dept. of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh for further investigation.

2.2. Isolation and optimization of coliform bacterial strains

Separately, each sample was used for plating on selective media. In this case, MacConkey agar media (Peptones 3.000 mg/L; Pancreatic digest of gelatin 17.000 mg/L; Lactose monohydrate 10.000 mg/L; Bile salts 1.500 mg/L; Sodium chloride 5.000 mg/L; Crystal violet 0.001 mg/L; Neutral red 0.030 mg/L; Agar 13.500 mg/L) was used. Plates were incubated at 37°C for 24 hour. Then, coliform bacteria were identified on the basis of colony morphology and lactose fermenting ability. Finally, isolates were maintained as pure cultured strains and preserved for further experiments. The effects of pH and temperatures on growth of isolated bacteria were optimized at 660 nm wavelength using spectrophotometer (Analytik Jena, Germany).

2.3. Morphological and biochemical characterization of isolated bacteria

Morphological and biochemical tests were done for the specific identification and characterization of bacteria. Isolated bacteria were characterized by several morphological (Gram staining and motility) and biochemical (Methyl red, catalase, starch agar, mannitol salt agar, TSI, simmons citrate agar and urea agar) tests.

2.4. Molecular identification of isolated bacteria

Molecular identification and characterization of the isolates were performed through the following steps: extraction of chromosomal DNA,[25] amplification of 16S rRNA

gene sequence, purification of PCR products, cycle sequencing, purification of cycle sequencing products, detection of nucleotides and sequence analysis. 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used as forward and reverse primer, respectively. 16S rRNA gene sequences of selected bacterial isolates were compared with other reference sequences as available in the NCBI database using the Basic Local Alignment Search Tool (BLAST) algorithm.

2.5. Antibiotic sensitivity test and determination of minimum inhibitory concentration (MIC)

Antibiotic sensitivity and resistance of the isolated bacteria were assayed according to the Kirby- Bauer disc diffusion method.[26] It was done against penicillin, amoxicillin, erythromycin, ampicillin, kanamycin, ceftazidimde, doxycycline, gentamycin, tetracycline, ciprofloxacin, cefuroxime and cefixime, respectively. Zones of inhibition were measured with the help of mm scale.

Minimum Inhibitory Concentration (MIC) of isolated bacteria was carried out through tube dilution method.[27]

2.6. Assessing toxicity of isolated bacteria on the developmental stages of Artemia salina

Artemia salina is commonly used for toxicity tests owing to its extensive distribution, short life cycle, non-selective grazing and sensitivity to toxic substances.[28] Acute toxicity assays were performed with *Artemia* cysts in two phases such as stationary (up to 48 hour) and exponential (up to 8 hour) to assess differences in cell toxicity of coliform bacterial strains isolated from drinking water.

2.7. Stationary phase

The stationary phase of *Artemia* cysts was maintained up to 48 hour at appropriate cyst germination laboratory condition (25°C and aeration pump) to assure well germination with 3 replications and control. Germination of cysts with each bacteria (O.D. = 0.5) and controls with triplicate were observed under inverted microscope (LABOMED CXL, USA) after every six hours of incubation. Data were kept and photographs of abnormalities were taken under a microscope (LABOMED CXL, USA).

2.8. Exponential phase

The exponential phase of *Artemia salina* was maintained up to 8 hour at appropriate laboratory condition (25°C and aeration pump) with 3 replications and control. Intoxication assays were performed in 6-well plates where each well contains 10 ml of filtered seawater, six *Artemia salina.* Then, the well plates were incubated for 8 hour with overnight cultured isolated bacteria (O.D.= 0.5) in abundances of 200 cells ml^{-1}. Each treatment containing the brine shrimps and one isolated bacteria was performed in triplicate and the control without the isolated bacteria was also maintained in triplicate. Abnormalities and survival of individuals were verified under inverted microscope (LABOMED CXL, USA) after every hour of incubation.

2.9. Determination of tD_{50}

The tD_{50} value was also determined in exponential phase. It is the time (hour) when 50% *Artemia salina* died in case of exponential phase.

2.10. Statistical analysis

Statistical analysis was done using Statistical Analysis Software (SAS), version 9.1.3 by Duncan Multiple Range Test (DMRT).

2.11. Determination of LC_{50}

Toxicity of three isolated bacteria were evaluated through detection of LC_{50} at concentrations of 25 μl, 50 μl, 75 μl, 100 μl, 125 μl, 150 μl**.** At first, simulated sea water was taken in a small tank and shrimp eggs (1.5 gm/l) were added to one side of the perforated divided tank with constant oxygen supply to get nauplii. Finally, 10 ml of simulated sea water solution with 10 nauplii was added to each of the test tube. The test tubes were left uncovered under the lamp and an incubation period of 24 hours was given at room temperature for observation. For each concentration, one vial containing 10 ml sea water and 10 shrimp nauplii were used as positive control group. It was used to verify the validity of the test. After 24 hours, the vials were observed. The number of survived nauplii in each vial was counted and the results were noted. The probit analysis was carried out through the Finney method to determine LC_{50}.[29]

3. Results

3.1. Isolation of coliform bacteria and optimization at different pH and temperature

Among twelve samples, highest number of coliform colonies were found from Sample 1 (local drinking water) then Sample 7 (bottled water), Sample 9 (bottled water) and were selected for further investigation on the basis of colony number and morphology but Sample 5 (bottled water) showed no growth on MacConkey agar media (Figure 1). Isolate A (*Aeromonas* sp.) showed highest growth at pH 7.2 and 35°C (Figures 2 and 3); Isolate B (*Enterobacter* sp.) showed maximum growth at pH 7.0 and 30°C (Figures 4 and 5) while Isolate C (*Escherichia* sp.) revealed utmost growth at pH 7.4 and 35°C (Figures 6 and 7).

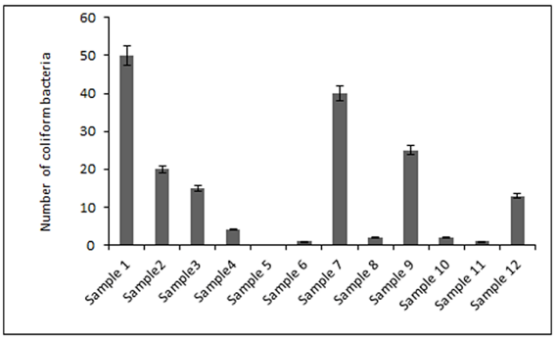

Fig. 1: Availability of coliform bacteria in twelve samples

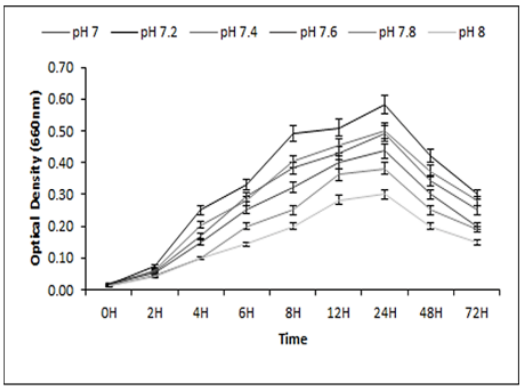

Fig. 2: Effect of optimum pH level on the growth of Isolate A (*Aeromonas* sp.) upto 72 hours of incubation period

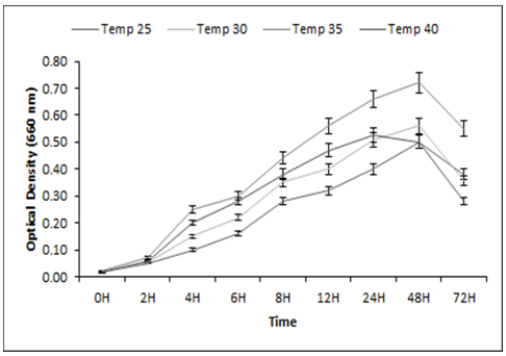

Fig. 3: Effect of temperature variations on the growth of Isolate A (*Aeromonas* sp.) upto 72 hours of incubation period

3.2. Morphological and biochemical characterization of isolated bacteria

Morphological characteristics indicated that all three isolates were motile, gram negative, rod shaped bacteria. Biochemical tests confirmed that isolate A was lactose fermenting, gas non-producing; methyl red, catalase and starch hydrolysis test positive; simmmons citrate, urea hydrolysis and mannitol salt agar test negative. Isolate B was lactose non- fermenting, gas producing; catalase,

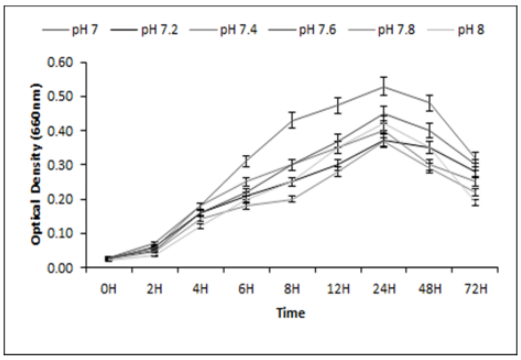

Fig. 4: Effect of optimum pH level on the growth of Isolate B (*Enterobacter* sp.) upto 72 hours of incubation period

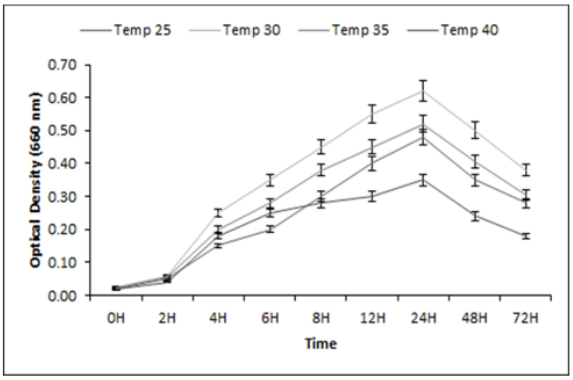

Fig. 5: Effect of temperature variations on the growth of Isolate B (*Enterobacter* sp.) upto 72 hours of incubation period

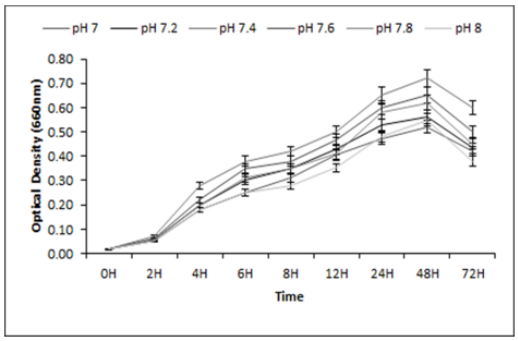

Fig. 6: Effect of optimum pH level on the growth of Isolate C (*Escherichia* sp.) upto 72 hours of incubation period

simmons citrate, urea hydrolysis and starch hydrolysis test positive; methyl red and mannitol salt agar test negative while isolate C showed positive result in all tests.

3.3. Molecular identification of isolated bacteria

When the 16S rRNA gene sequences of isolated bacteria were verified with the 16S rRNA gene sequences of other organisms that had already been submitted to NCBI Gene

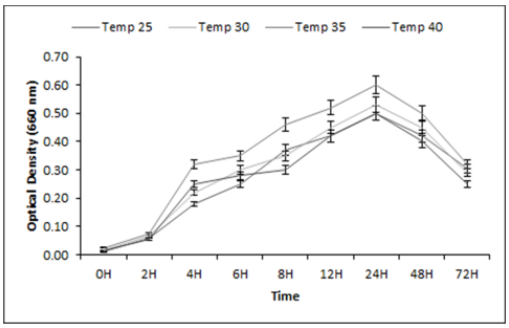

Fig. 7: Effect of temperature variations on the growth of Isolate C (*Escherichia* sp.) upto 72 hours of incubation period

bank database using the BLASTN (http://www.ncbi.nih.gov /BLAST) algorithm, it indicated that isolate A showed 76% identity with *Aeromonas* sp. (Accession no. LC435695), isolate B showed 96% identity with *Enterobacter* sp. (Accession no. LC434449) while isolate C showed 96% identity with *Escherichia* sp. (Accession no. LC384629). DNA quantification analysis and PCR band of isolated bacteria were shown in Figures 8 and 9.

3.4. Antibiotic sensitivity test and determination of minimum inhibitory concentration

The result showed that, *Aeromonas* sp. and *Escherichi*a sp. were multi drug resistant. *Aeromonas* sp. was resistant against penicillin, ceftazidime, doxycycline, cefuroxime and cefixime (Table 1). However, *Escherichia* sp. was resistant against penicillin, ceftazidime, doxycycline and cefuroxime (Table 1). On the other hand, *Enterobacter* sp. was resistant against ceftazidime and cefuroxime (Table 1).

3.5. Assessing toxicity of isolated bacteria on the developmental stages of Artemia salina

3.6. Stationary phase

After 16 hour, all three isolates, *Aeromonas* sp., *Enterobacter* sp. and *Escherichia* sp. treated *Artemia* cyst showed no germination (Figure 10). After 24 and 36 hour, *Aeromonas* sp. and *Enterobacter* sp. treated cyst showed germination but *Escherichia* sp. treated cyst showed no germination and the variation was significant between the treatments compared to control (Figure 10). However, after 48 hour, all the isolates showed germination and the variation was significant among control with all three bacteria treated cyst (Figure 10). Major abnormalities were observed under microscope (LABOMED CXL, USA) which was shown in Figure 11.

The minimum inhibitory concentrations of *Aeromonas* sp., *Enterobacter* sp. and *Escherichia* sp. against gentamycin were 6.25 μg/ml, 12.5 μg/ml and 6.25 μg/ml, respectively (Table 2).

The minimum inhibitory concentrations of *Aeromonas* sp., *Enterobacter* sp. and *Escherichia* sp. against amoxicillin were observed 1.56 μg/ml, 12.5 μg/ml and 3.125 μg/ml, respectively (Table 3).

3.7. Exponential phase

Toxic effects of the three coliform isolates were conducted on germinated *Artemia salina* and treatment was added with three replications for each bacterium as well as control maintained. After 1 hour, all isolated bacteria treated *Artemia* showed highest level of survival and the variation was non-significant among the treatments in comparison to control (Figure 12). After 2 hour, bacteria treated *Artemia* showed low survival than 1 hour and the variation was significant among treatments compared to control (Figure 12). Again, after 3-7 hour, we found that the survival of *Artemia* was decreased with time and the variation was risen significantly in comparison to control (Figure 12). However, after 8 hour, no survival was found in case of treatment with *Escherichia* sp. and the variation was significant. Major abnormalities were also observed under microscope (LABOMED CXL, USA) which was shown in Figure 13.

3.8. Determination of tD_{50}

tD_{50} value was observed in case of exponential phase which were 6 hour, 7 hour and 4 hour for *Aeromonas* sp., *Enterobacter* sp. and *Escherichia* sp., respectively (Table 4).

3.9. Determination of LC_{50}

Isolated *Aeromona*s sp., *Enterobacter* sp. and *Escherichia* sp. showed 50% toxicity against aquatic organism at the concentration of 56.26$\pm$0.18, 85.41$\pm$0.23 and 48.32$\pm$0.17 μl, respectively (Table 5). Regression line of log dose and probit mortality of isolated bacteria against brine- shrimp nauplii was shown in Figures 14, 15 and 16.

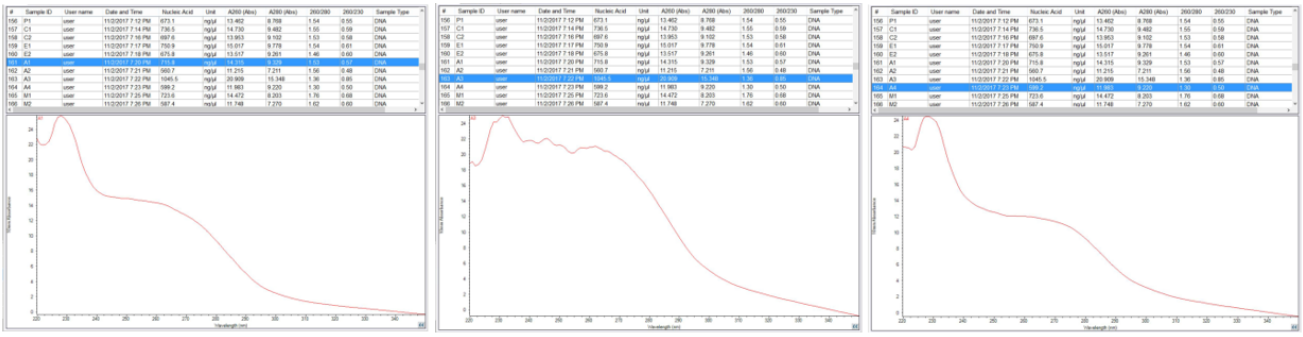

Fig. 8: DNA quantification analysis of isolate A, isolate B and isolate C

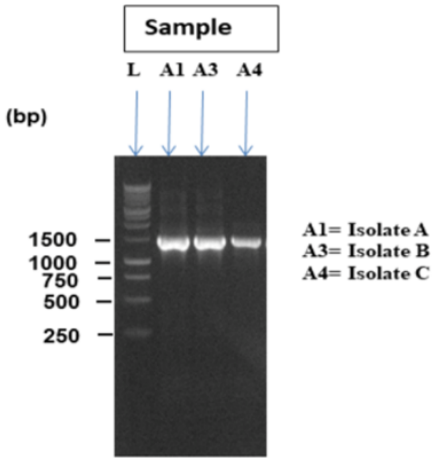

Fig. 9: 16S rRNA gene profiling of isolated bacteria A1 (Isolate A), A3 (Isolate B) and A4 (Isolate C) using 27F and 1492R primers, L denotes 1kb DNA ladder (marker)

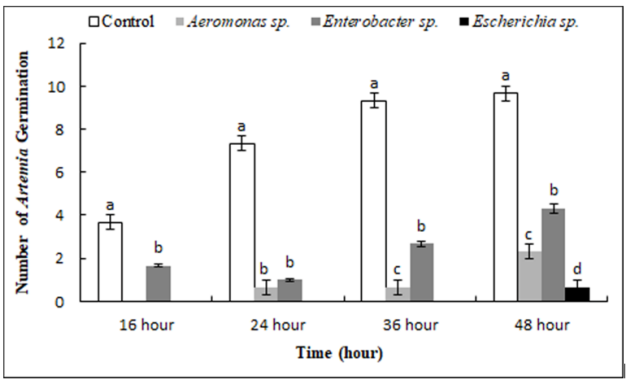

Fig. 10: Variation of *Artemia salina* germination number through DMRT at stationary phase

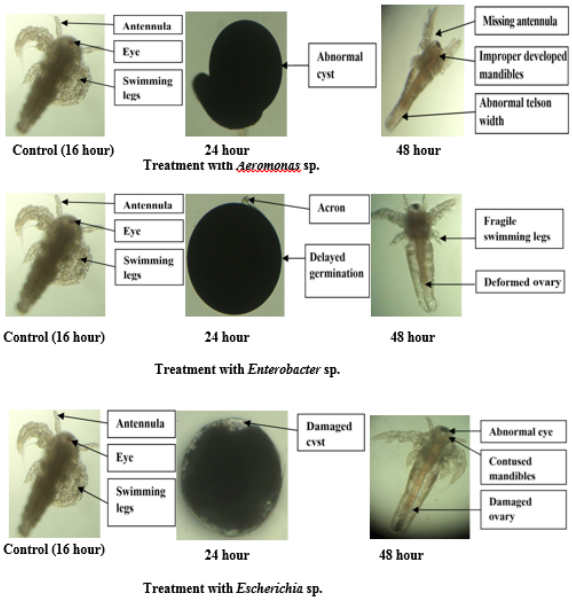

Fig. 11: Toxic effects of isolated coliform bacteria on *Artemia salina* at stationary phase

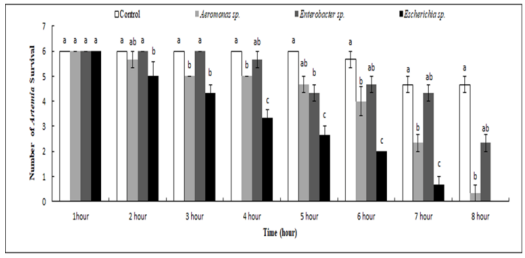

Fig. 12: Variation of *Artemia salina* survival number through DMRT at exponential phase

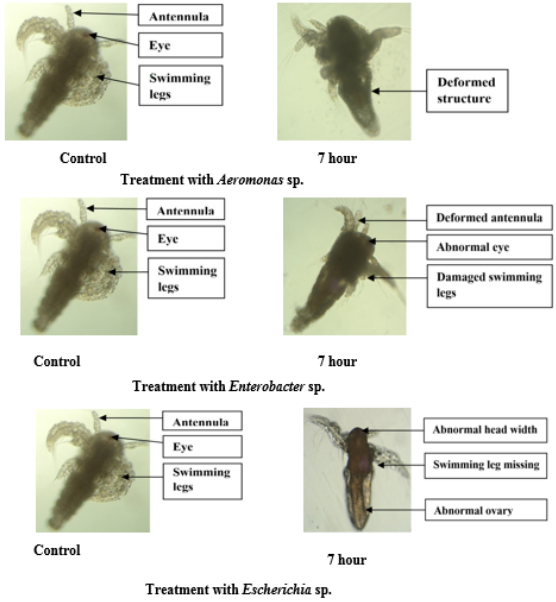

Fig. 13: Toxic effects of isolated coliform bacteria on *Artemia salina* at exponential phase

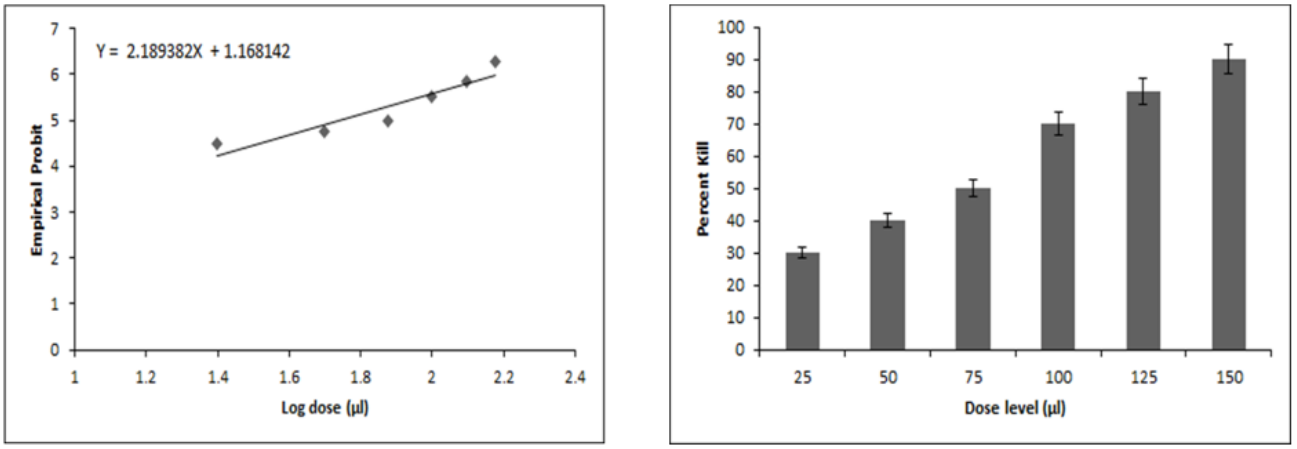

Fig. 14: Regression line of log dose and probit mortality of *Aeromonas* sp. against brine-shrimp nauplii after 24 hours of exposure

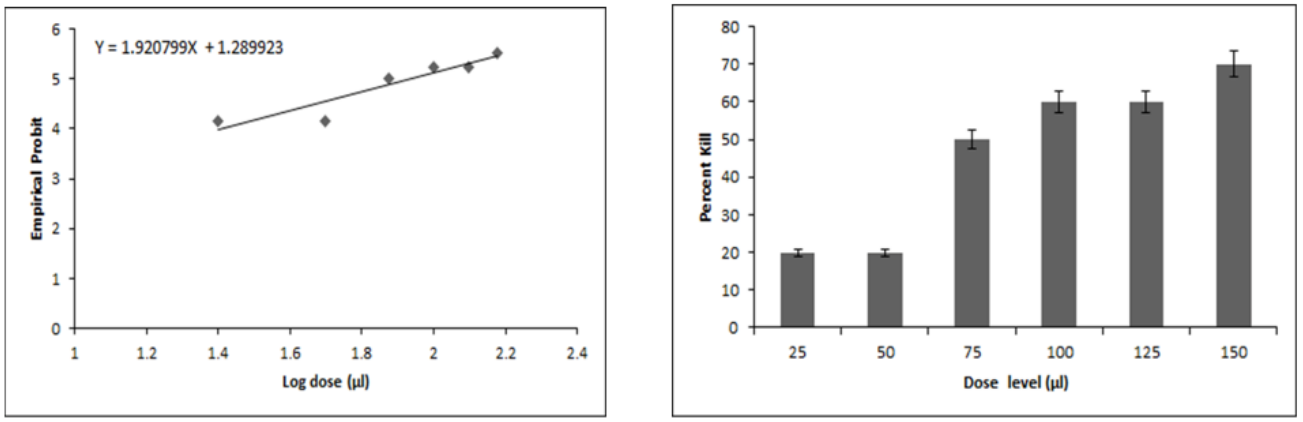

Fig. 15: Regression line of log dose and probit mortality of *Enterobacter* sp. against brine-shrimp nauplii after 24 hours of exposure

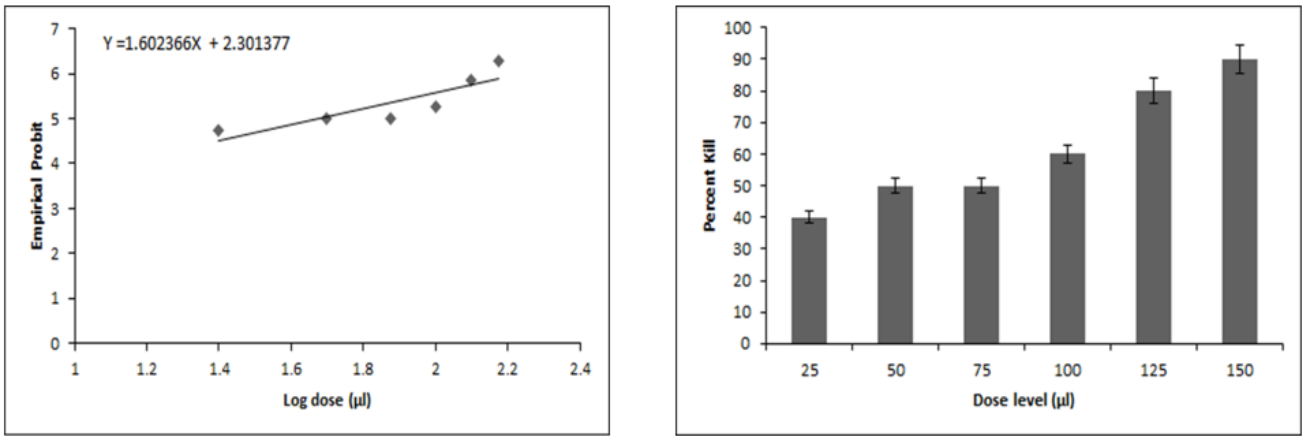

Fig. 16: Regression line of log dose and probit mortality of *Escherichia* sp. against brine-shrimp nauplii after 24 hours of exposure

Table 1: Antibiotic sensitivity test used for the detection of the resistance pattern of the isolated bacteria

Name of Antibiotic	Zone of inhibition (mm)			Resistant pattern		
	Aeromonas sp.	*Enterobacter* sp.	*Escherichia* sp.	*Aeromonas* sp.	*Enterobacter* sp.	*Escherichia* sp.
Penicillin	10 mm	14 mm	8 mm	Resistant	Intermediate resistant	Resistant
Amoxicillin	20 mm	16 mm	16 mm	Susceptible	Susceptible	Susceptible
Erythromycin	17 mm	30 mm	28 mm	Susceptible	Susceptible	Susceptible
Ampicillin	15 mm	15 mm	15 mm	Intermediate resistant	Intermediate resistant	Intermediate resistant
Kanamycin	17 mm	20 mm	19 mm	Susceptible	Susceptible	Susceptible
Ceftazidimde	7 mm	8 mm	8 mm	Resistant	Resistant	Resistant
Doxycycline	9 mm	22 mm	10 mm	Resistant	Susceptible	Resistant
Gentamycin	17 mm	19 mm	17 mm	Susceptible	Susceptible	Susceptible
Tetracycline	17 mm	23 mm	22 mm	Susceptible	Susceptible	Susceptible
Ciprofloxacin	22 mm	25 mm	25 mm	Susceptible	Susceptible	Susceptible
Cefuroxime	9 mm	10 mm	6 mm	Resistant	Resistant	Resistant
Cefixime	8 mm	14 mm	13 mm	Resistant	Intermediate resistant	Intermediate resistant

Note: Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15 mm

Table 2: The minimum inhibitory concentration of isolated bacteria against gentamycin

Test Organism	Growth response at different concentrations								
	Gentamycin (μg/ml)								
Aeromonas sp.	100-	50-	25-	12.5-	6.25-	3.125+	1.56+	0.78+	0.39+
	Gentamycin (μg/ ml)								
Enterobacter sp.	100-	50-	25-	12.5-	6.25+	3.125+	1.56+	0.78+	0.39+
	Gentamycin (μg/ml)								
Escherichia sp.	100-	50-	25-	12.5-	6.25-	3.125+	1.56+	0.78+	0.39+

Note: The '+' sign indicates the growth of the microorganisms while '-' sign indicates no growth

Table 3: The minimum inhibitory concentrations of isolated bacteria against amoxicillin

Test Organism	Growth response at different concentrations								
Aeromonas	Amoxicillin (μg/ml)								
sp.	100-	50-	25-	12.5-	6.25-	3.125-	1.56-	0.78+	0.39+
Enterobacter	Amoxicillin (μg/ml)								
sp.	100-	50-	25-	12.5-	6.25+	3.125+	1.56+	0.78+	0.39+
Escherichia	Amoxicillin (μg /ml)								
sp.	100-	50-	25-	12.5-	6.25-	3.125-	1.56+	0.78+	0.39+

Note: The '+' sign indicates the growth of the microorganisms while '-' sign indicates no growth

Table 4: Time (hr) when 50% *Artemia salina* died (tD_{50}) in exponential phase

Growth Phase	(*Aeromonas* sp.)	(*Enterobacter* sp.)	(*Escherichia* sp.)
Exponential	6	7	4

Table 5: LC_{50}, 95% confidence limits, regression equations and Chi-square values for isolated bacteria against brine shrimp nauplii after 24 hours of exposure

Test sample	LC50 (μl)	95% Confidence limits (μl)	Regression equation	χ2 value (Degrees of freedom)
Aeromonas sp.	56.26±0.18	37.89 to 83.52	Y =2.189x+1.168	1.508 (4)
Enterobacter sp.	85.41±0.23	57.16 to 127.65	Y =1.920x+1.289	1.239 (4)
Escherichia sp.	48.32±0.17	27.03 to 86.36	Y =1.602x+2.301	2.136 (4)

4. Discussion

Several studies have reported isolation and characterization of coliform bacteria from drinking water. Parvez et al. outlined bacteriological quality of drinking water samples a cross Bangladesh.[30] Ahmed et al. also published article on the isolated fecal indicators and bacterial pathogens in bottled water from Dhaka, Bangladesh.[31] However, our study was designed not only to coliform bacterial strains (Isolate A as *Aeromonas* sp., Isolate B as *Enterobacter* sp. and Isolate C as *Escherichia* sp.) isolation, physiological study and profiling resistance pattern but also for the evaluation of their acute toxicity on the developmental stages of aquatic organism especially on *Artemia salina*. Morphological identification showed all three isolates were motile, gram negative and rod shaped bacteria. Biochemical characteristics indicated that isolate A was lactose fermenting, gas non-producing, methyl red, catalase and starch hydrolysis test positive; isolate B was lactose non- fermenting, gas producing, catalase, simmons citrate, urea hydrolysis and starch hydrolysis test positive while isolate C confirmed positive result for all tests. In our investigation, all three isolates illustrated comparative differences in the growth performances. They showed maximal growth at different pH and temperature. However, Isolate A revealed maximum growth at pH 7.2 and in 35°C, Isolate B at pH 7.0 and in 30°C while Isolate C at pH 7.4 and in 35°C. Following Bergey's Manual of Systematic Bacteriology[32] (Second Edition), isolate A showed similar characteristics of the genus *Aeromonas*, isolate B showed similar features of the genus *Enterobacter* while isolate C revealed similarity of the genus *Escherichia.* So, it can be assumed that outputs of physiological and biochemical features of the isolates have similarity with the referred data.

Comparison of the bacterial 16S rRNA gene sequencing has materialized as a preferred genetic technique in molecular biology.[33] Thus, in the present research, sequencings were done by 16S rRNA gene for molecular identification of the isolated bacterial strains. Sequencing result of 16S rRNA gene using the Basic Local Alignment Search Tool (BLAST) algorithm authenticated that the bacterial isolate A showed 76% significant alignments with *Aeromonas* sp., isolate B showed 96% significant alignments with *Enterobacter* sp. strain and isolate C showed 96% significant alignments with *Escherichia* sp. Merih et al. monitored the occurrence of pathogenic *Aeromonas* sp. in publi c drinking water.[34] Arun et al. also investigated the exsistence of enteric pathogens such as *Escherichia coli* and *Enterobacter aerogenes* from different drinking water sources.[35] Our sequencing results showed resemblance with the previously isolated coliforms.

Availability of multiple drug resistance (MDR) coliform was reported in tubewell water.[36] They found that all of the coliform isolates were resistant to ampicillin, all fecal coliform isolates to penicillin and sulphamethoxazole. Another study of quality analysis of Dhaka WASA drinking water by Mahbub et al. showed that all eight *E. coli* isolates were found resistant to ampicillin, amoxicillin, kanamycin, penicillin antibiotics and almost all of them were found sensitive to gentamycin, nalidixic acid and ciprofloxacin.[37] Beside these, Scoaris et al.reported multiple drug resistance *Aeromonas* sp.[38] and prevalence of multidrug resistant *Escherichia coli* from drinking water sources were also investigated.[39] Antibiotic resistant *Enterobacter* sp. was also found from raw source water and treated drinking water.[40] In the present study, multiple drug resistance (MDR) isolates were found. Among three isolates, *Aeromonas* sp. and *Escherichia* sp. exhibited resistance to multiple antibiotics while *Enterobacter* sp. was resistant against only two antibiotics. *Aeromonas* sp. was resistant to penicillin, ceftazidime, doxycycline, cefuroxime, cefixime and *Escherichia* sp. was resistant to penicillin, ceftazidime, doxycycline and cefuroxime but *Enterobacter* sp. was resistant to only ceftazidime and cefuroxime. Thus, the resistance was showed as *Aeromonas* sp.> *Escherichia* sp.> *Enterobacter* sp. in this pattern. All the isolates were susceptible to amoxicillin, erythromycin, kanamycin, gentamycin, tetracycline and ciprofloxacin. Moreover, the MIC value of gentamycin against *Aeromonas* sp., *Enterobacter* sp. and *Escherichia* sp. were 6.25 μg/ml, 12.5 μg/ml and 6.25 μg/ml, respectively. The MIC value of another antibiotic amoxicillin was also determined and the MIC concentrations for the *Aeromonas* sp., *Enterobacter* sp. and *Escherichia* sp. were found as 1.56 μg/ml, 12.5 μg/ml and 3.125 μg/ml, respectively. From the test, it was confirmed that between these two antibiotics, *Aeromonas* sp. and *Escherichia* sp. can be easily controlled by amoxicillin (β-lactum class) in contrast to gentamycin (amino glycosides class). Our result confirmed promising outcome to control coliforms in future aspect.

Supratik Kar et al. proposed interspecies cytotoxicity parallel models between *Escherichia coli* (prokaryotic system) and human cell line (HaCaT) (eukaryotic system).[41] Previously, Neves et al. reported acute toxicity of dinoflagellate cells in two growth phases of *Artemia salina* at 200 cells ml^{-1} to assess differences in cell toxicity to *Artemia salina*.[42] In their study, the toxic benthic dinoflagellates significantly affected the mortality and survival rates of *Artemia salina* in stationary and exponential phase, respectively. Highest tD_{50} value was also found 4 hour for brine shrimps depicted to G. excentricus in exponential phase. Notably, we performed bacterial toxicity assays on *Artemia salina* in stationary and exponential phase with 200 cells ml^{-1} of isolated bacteria. No previous data about the toxic effect of coliform bacteria on the developmental stages of *Artemia salina* was reported before our investigation. In case of stationary phase, all bacterial treated cysts showed preferably delayed germination. In addition to that other abnormalities were detected among

bacteria treated germinated cysts such as missing antennula, improper developed mandibles, abnormal telson width were photographically recorded when treated by *Aeromonas* sp. and fragile swimming legs, deformed ovary were captured by *Enterobacter* sp. treatment and abnormal eye, contused mandibles, damaged ovary were confined by *Escherichia* sp. treatment. Our results also illustrated significant level of developmental abnormalities at exponential phase of *Artemia salina* by the isolates in comparison to control. Deformed structure was observed in case of *Aeromonas* sp. treatment; deformed antennula, abnormal eye, damaged swimming legs were noticed in case of *Enterobacter* sp. treatment; abnormal head width, swimming leg missing, abnormal ovary were found in case of *Escherichia* sp. treatment. The tD_{50} was also experimented in exponential phase. In case of *Aeromonas* sp., tD_{50} was found within 6 hour, *Enterobacter* sp. within 7 hour while *Escherichia* sp. within 4 hour which showed similarity with referred data. Remarkably, variation in germination and survival of *Artemia salina* were increased with time among all three treatments in comparison to control and it was confirmed by DMRT analysis. From the result, it was clear that highest level of toxicity was observed in case of *Escherichia* sp. in both phases (stationary and exponential).

In our study, the LC_{50} results of the isolates were also evaluated. LC_{50} values of *Aeromonas* sp., *Enterobacter* sp. and *Escherichia* sp. were 56.26±0.18 m l, 85.41±0.23 μl and 48.32±0.17 μl after 24 hours, respectively. Harwig and Scott described 50% mortality (LC_{50} value) of known mycotoxins for 16 hour against *Artemia salina* which was 1.3 μg/ml for aflatoxin G1, 3.5 μg/ml for gliotoxin and 10.1 μg/ml for ochratoxin A.[43] From our data, it was clear that the bacterial isolates had cytotoxic effect against the aquatic organism *A. salina* and the toxicity level was marked as *Escherichia* sp.$>$ *Aeromona*s sp. $>$ *Enterobacter* sp. after 24 hour of exposure on *Artemia salina* which showed less toxicity than the referred toxins data.

5. Conclusion

The antibiotic resistance patterns of these isolated gram-negative bacterial strains are correlated well, perhaps indicating their common origin and mode for emerging as antibiotic resistance strains. On the other hand, DMRT analysis indicated significant abnormalities in *Artemia salina* caused by multidrug coliforms and enunciate that isolated bacteria would have harmful effect for human health which can cause serious water borne diseases. Therefore, development and spread of antibiotic resistance in bacteria should pay attention while prescribing antibiotics to treat patients suffering from infection caused by pathogenic bacteria isolated from the studied samples.

6. Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Gomes DF, Rassan DR, Luis MB, Mnica SB, Lilian CC. Bacteriological water quality in schools drinking fountains and detection of antibiotic resistance genes. *Ann Clin Microbiol Antimicrob*. 2017;16(1):5–5.
2. Sarkar BL, Roy MK, Chakrabarti AK, Niyogi SK. Distribution of phage type Vibrio cholera Ol biotype El Tor in Indian scenario (1991-98). *Indian J Med Res*. 1999;109:204–207.
3. Gruber JS, Ercumen A, Colford JM. Coliform bacteria as indicators of diarrheal risk in household drinking water: Systematic review and meta-analysis. *PLoS One*. 2014;9:107429.
4. Isfahani BN, Fazeli H, Babaie Z, Poursina F, Moghim S, Rouzbahani M. Evaluation of polymerase chain reaction for detecting coliform bacteria in drinking water sources. *Adv Biomed Res*. 2017;6:130.
5. Mcfeters GA, Cameron SC, Chevallier MWL. Influence of diluents, media, and membrane filters on detection of injured waterborne coliform bacteria. *Appl Environ Microbiol*. 1982;43(1):97–103.
6. Bafghi MF. Phenotypic and molecular identification of nocardia in brain abscess. *Adv Biomed Res*. 2017;6:49.
7. Seas C, Alarcon M, Aragon JC, Beneit S, Quiñonez M, et al. Surveillance of bacterial pathogens associated with acute diarrhea in Lima, Peru. *Int J Infect Dis*. 2000;4(2):96–99.
8. Walker CLF, Rudan I, Liu L, Nair H, Theodoratou E, et al. Global burden of childhood pneumonia and diarrhea. *Lancet*. 2013;381(9875):1405–1416.
9. World Health Organization, Progress towards the millennium development goals, 1990-2005, 2005, World Health Organization, Geneva, Switzerland.
10. Joo PSC. Water microbiology: Bacterial pathogens and water. *Int J Environ Res Public Health*. 2010;7(10):3657–3703.
11. Hossain MF. Arsenic contamination in Bangladesh- an overview. *Agr Ecosyst Environ*. 2006;113(1-4):1–16.
12. Islam MA, Sakakibara H, Karim MR, Sekine M, Mahmud ZH. Bacteriological assessment of drinking water supply options in coastal areas of Bangladesh. *J Water Health*. 2011;9(2):415–428.
13. United Nations International Children's Emergency Fund (1995). Progotir Pathey Bangladesh: Bangladesh Bureau of Statistics.
14. Islam MA, Sakakibara H, Karim MR, Sekine M, Mahmud ZH. Bacteriological assessment of drinking water supply options in coastal areas of Bangladesh. *J Water Health*. 2011;9(2):415–428.
15. Humphrey JH. Child undernutrition, tropical enteropathy, toilets and handwashing. *Lancet*. 2009;374:1032–1035.
16. Stephen PL, Mahbubur R, Benjamin FA, Leannie U, Sania A, Peter JW. Effects of water quality, sanitation, handwashing and nutritional interventions on diarrhoea and child growth in rural Bangladesh: a cluster randomised controlled trial. *The Lancet Glob Health*. 2018;6:302–315.
17. Bartram J, Cotruvo J, Exner M, Fricker C, Glasmacher A. Heterotrophic plate count measurement in drinking water safety management: report of an expert meeting Geneva. *Int J Food Microbiol*. 2002;92(3):241–247.
18. Jamir M. Accessing safe drinking water. The Daily Star, Sep 26, 2009. Dhaka, Bangladesh. In: Accessing safe drinking water. The Daily Star.
19. Venieri D. Microbiological evaluation of bottled non-carbonated (still) water from domestic brands in Greece. *Int J Food Microbiol*. 2006;107(1):68–72.
20. Svagzdiene R, Lau R, Page RA. Microbiological quality of bottled water brands sold in retail outlets in New Zealand. *Water Sci Technol*. 2010;10(5):689–699.
21. Khan AA, Cerniglia CE. Detection of *Pseudomonas aeruginosa* from clinical and environmental samples by amplification of the exotoxin A gene using PCR. *Appl Environ Microbiol*. 1994;60(10):3739–3745.
22. Warburton DW, Bowen B, Konkle A. The survival and recovery of Pseudomonas aeruginosa and its effect upon salmonellae in water: Methodology to test bottled water in Canada. *Can J Microbiol*. 1994;40(12):987–992.

23. Blake PA, Rosenberg ML, Florencia J. Cholera in Portugal, 1974. II. Transmission by bottled mineral water. *Am J Epidemiol*. 1977;105(4):344–348.
24. World Health Organization, Guidelines for Drinking Water Quality: Incorporating 1st and 2nd Addenda, 2008, Vol.1, Recommendations, 3rd ed. World Health Organization, Geneva, Switzerland.
25. Sambrook J, Fritschi EF, Maniatis T. Molecular cloning: a laboratory manual 1989. Cold Spring Harbor Laboratory Press, New York.
26. Bauer AW, Kirby WM, Sherris JC, Turek M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol*. 1966;45(4):493–496.
27. Lawrence AC. Tube dilution antimicrobial susceptibility testing: Efficacy of a microtechnique applicable to diagnostic laboratories. *Appl Microbiol*. 1969;17(5):707–709.
28. Faimali M, Giussani V, Piazza V, Garaventa F, Corr C, Asnaghi V. Toxic effects of harmful benthic dinoflagellate Ostreopsis ovate on invertebrate and vertebrate marine organisms. *Mar Environ Res*. 2012;76:97–107.
29. Finney DJ. Probit analysis. 3rd ed Cambridge: Cambridge University Press ; 1971; pp. 333.
30. Parvez AK, Liza SM, Marzan M. Bacteriological quality of drinking water samples across Bangladesh. *Arc Clin Microbiol*. 2016;7:1.
31. Ahmed W, Yusuf R, Hasan I, Ashraf W, Goonetilleke A, Toze S. Fecal indicators and bacterial pathogens in bottled water from Dhaka, Bangladesh. *Braz J Microbiol*. 2013;44(1):97–103.
32. Bergeys Mannual of Systematic Bacteriology. The Proteobacteria Part B, Second Edition, Volume Two, 2006; pp. 557-611.
33. Clarridge JE. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin Microbiol Rev*. 2004;17(4):840–862.
34. Merih K, Meral Y, Filiz D. The Occurrence of Aeromonas in drinking water, tap water and the Porsuk river. *Braz J Microbiol*. 2011;42(1):126–131.
35. Arun K, Aradhana D, Aminul M. Microbial analysis of potable water and its management through useful plant extracts. *Int J Sci Res*. 2017;73(3):44–49.
36. Datta RR, Hossain MS, Aktaruzzaman M, Fakhruddin ANM. Antimicrobial resistance of pathogenic bacteria isolated from tubewell water of coastal area of Sitakunda. *Chittagong Open J Water Pollut Treat*. 2013;1(1):12–15.
37. Mahbub KR, Nahar A, Ahmed MM, Chakraborty A. Quality analysis of Dhaka WASA drinking water: detection and biochemical characterization of the isolates. *J Environ Sci Nat Res*. 2011;4(2):41–49.
38. Dde OS, Colacite J, Nakamura CV, Ueda-Nakamura T, Filho BADA, Filho BPD. Virulence and antibiotic susceptibility of *Aeromonas* sp. isolated from drinking water. *Antonie Van Leeuwenhoek*. 2008;93(1-2):111–122.
39. Chen Z, D Y, He S, Zhang L, Wen Y, et al. Prevalence of antibiotic-resistant *Escherichia coli* in drinking water sources in Hangzhou city. *Front Microbiol*. 2017;8:1133.
40. Scott B, Raj B, Rajkumar N, Angie C, Gary L. Presence of antibiotic resistant bacteria and antibiotic resistance genes in raw source water and treated drinking water. *Int Biodeterior Biodegr*. 2015;102:370–374.
41. Supratik K, Agnieszka G, Kunal R, Jerzy L, Tomasz P. Extrapolating between toxicity endpoints of metal oxide nanoparticles: Predicting toxicity to Escherichia coli and human keratinocyte cell line (HaCaT) with nano-QTTR. *Ecotoxicol Environ Saf*. 2016;126:238–244.
42. Neves R, Fernandes T, Santos LN, Nascimento SM. Toxicity of benthic dinoflagellates on grazing, behavior and survival of the brine shrimp *Artemia salina*. *PLoS One*. 2017;12:175168–175168.
43. Harwig J, Scott P. Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. *Appl Microbiol*. 1971;21(6):1011– 1016.

Author biography

Afrin Priya Talukder Student

Md. Nazmul Haque Student

Rashed Zaman Associate Professor

Md. Akhtar-E- Ekram Associate Professor

Cite this article: Priya Talukder A, Haque MN, Zaman R, Ekram MAE. Profiling resistance pattern and assessing toxicity of coliform bacteria on the developmental stages of *Artemia salina* isolated from drinking water of northern part of Bangladesh. *Indian J Microbiol Res* 2019;6(4):355-366.