J. Bio-Sci. 32(2): 23-30, 2024 **ISSN 1023-8654**
http://www.banglajol.info/index.php/JBS/index
DOI:https://doi.org/10.3329/jbs.v32i2.75754

DETECTION AND DIVERSITY OF TETRACYCLINE RESISTANT BACTERIA IN FISH PONDS OF RAJSHAHI REGION

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Abstract

The growth of aquaculture as a key food production industry, particularly in Bangladesh, has led to the increased use of various chemicals and drugs. This study focuses on the critical analysis of bacterial profiles in the waters of fish farming ponds in the Rajshahi district of Bangladesh, a necessary step for disease management and addressing the public health issues related to antibiotic-resistant bacteria. The research found a significant presence of bacteria in these waters, with viable bacterial counts fluctuating from 3.0×10^2 to 4.2×10^3 CFU/ml at a Tetracycline concentration of 32 µg/ml, and 0.5×10^2 to 1.8×10^3 CFU/ml at 64 µg/ml. Using 16S rRNA gene analysis, we discerned a dozen distinct bacterial species spanning six genera that demonstrate resistance to tetracycline. These include *Enterobacter asburiae*, *Aeromonas* sp., *Acinetobacter* sp., *Bacillus* sp., *Bacillus cereus*, *Enterobacter cloacae*, *Acinetobacter gandensis*, *Escherichia coli*, *Shigella* sp., *Enterobacter hormaechei*, *Acinetobacter baumannii*, and *Bacillus altitudinis*. The study underscores the critical need for preventive actions in fish farming to address these resistant bacteria and calls for comprehensive research to trace their origins and pathways of transmission. This research significantly contributes to the understanding of antibiotic resistance in aquatic ecosystems and its broader implications for public health.

Key words: Antibiotic-resistance bacteria, Fish pond, Rajshahi region, Tetracycline, 16S rRNA gene analysis.

Introduction

Fish, a healthy dietary option rich in safe and high-quality protein with low fat content, provides approximately 60% of our daily animal protein consumption (Farzana et al. 2018). In Bangladesh, especially for the impoverished population, it serves as a primary or supplementary means of livelihood and income, playing a significant role in the overall economic landscape of the nation (Dey et al. 2008). Approximately 19.5 million individuals, comprising 1.4 million women, which accounts for 12% of the total population, rely on the fisheries sector for their livelihood (Farzana et al. 2018). The overall fish production in Rajshahi region was calculated at 59,864 metric tons (mt), with ponds contributing 35,302mt. Notably, ponds exhibited the highest fish production rate at 2,297 kg/ha (Mortuza and Hossain 2006). Fish are susceptible to illnesses triggered by a diverse range of bacterial pathogens (Bondad-Reantaso et al. 2023). Bacterial agents act as pathogens for both freshwater and marine fish. The bacteria are prevalent in fish and fish products obtained from both wild and cultivated ponds in various aquatic environments, representing the most frequently identified causes of fish diseases (Bondad-Reantaso et al. 2023). Antibiotics are employed to efficiently prevent and address bacterial infections (Brooks and Brooks 2014). The excessive utilization of antibiotics has led to the development of antibiotic resistance in fish pathogens, giving rise to antibiotic-resistant bacteria in aquatic ecosystems. Reports indicate that following administration, numerous antibiotics endure in sediment and the

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aquatic environment for extended periods, adversely impacting the microbial community within the sediment (Miranda and Zemelman 2002, Salma et al. 2022).

In recent times, the rise of bacteria resistant to multiple drugs presents a notable risk to both public health and environmental ecosystems (Huang et al. 2015, Asif et al. 2021). The aquatic setting provides an optimal environment for bacterial mutation, recombination, and horizontal gene transfer (Abe et al. 2020). Furthermore, the substantial public health concern arises from the multi-drug-resistant commensal *E. coli* strain, as *E. coli* serves as a proficient reservoir of antimicrobial resistance (AMR) genes, with the potential to transfer these genes to other bacteria (Dewi et al. 2022). Despite the significant role played by the Rajshahi district in pond-based aquaculture production in Bangladesh, limited research has focused on the indiscriminate use of various aqua-drugs, chemicals, and antibiotics in the finfish polyculture prevalent in this region. Consequently, this investigation was conducted to observe the prevalence of drug-resistant bacteria through 16S rRNA sequencing and determining the phylogenetic placement of these identified bacterial strains in pond water linked to fish farming in Rajshahi Region, Bangladesh. The outcomes of this study aim to offer scientific insights into the current state of antibiotic-resistant bacteria in the fish ponds in Rajshahi district of Bangladesh.

Materials and Methods

Sample collection and prospecting

Water samples were sourced from various fish ponds located in Rajshahi region (Table 1) during the period from January and February of Bangladesh. All the samples were collected and securely sealed in sterile falcon tube and placed in icebox. Following this, the samples were transported to the Laboratory within the Department of Zoology at the University of Rajshahi. They were expeditiously stored at a temperature of -20°C for further experiments. The topography of the sampling ponds is mentioned in Table 1.

Table 1: Topography of samples ponds.

Sample name	pH	Temperature (°C)
Rajshahi University-1 (RU-1)	8.0	21.7
Rajshahi University-2 (RU-2)	8.1	22.8
Baghmara (BAP)	8.0	24.0
Taherpur (TAP)	8.1	23.8
Rajpara (RAP)	8.5	23.0
Chapainawabgonj (CHP)	8.3	22.8
Gomostapur (GOP)	8.0	23.0
Kashiadanga (KAP)	8.3	22.8
Tanore (TNP)	7.9	24.0
Aradidhi (ARP)	7.8	23.2
Durgapur (DUP)	8.1	25.0
Puthia (PUP)	8.2	24.0

Determination of colony forming unit (CFU)

The quantification of colony forming units (CFUs) assumes a pivotal significance within the context of this experiment. CFUs serve as a metric to gauge the prevalence of precursor cells within a particular cell population. To determine the count of living bacteria, water samples were combined with sterile phosphate buffered saline (PBS) followed by a series of 10-fold dilutions. The enumeration was conducted on plates using nutrient broth with 1.5% nutrient agar supplemented with each of tetracycline (TC). Plates without any antibiotics served as the control group. These plates were then incubated at a temperature of 37°C. After 24 hours of incubation, the viable bacterial colonies were counted by following equation. The standard formula of CFU (Reasoner 2004) is given below:

$$\text{CFU} = \frac{\text{Number of colonies on agar plate}}{\text{Total dilution of tube} \times \text{amount plated}}$$

Extraction of DNA and PCR amplification

The DNA extraction from bacterial strains was conducted utilizing the Phenol-Chloroform method at the Central Dogma Lab., Invent Technologies Ltd., Dhaka, as previously delineated (Wright et al. 2017). Then, the 16S rRNA gene was amplified through polymerase chain reaction (PCR) on a Thermocycler. The PCR procedure was executed in 50 µL reaction volumes, comprising 2 µL of DNA template, 25 µL of 2x PCR Master Mix, and 2 µL of each primer (20 pmol/µL). The specific primers employed in this study were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and U1492R (5'-GGTTACCTTGTTACGACTT-3') (James 2010). The PCR amplification commenced with an initial DNA denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and elongation at 72°C for 1 minute, culminating with a final extension at 72°C for 5 minutes. The resulting PCR product (5 µL) was subjected to electrophoresis on a 1.5% agarose gel at 90 volts for 45 minutes. Subsequently, gel visualization was achieved via UV trans-illumination, and the results were duly recorded.

Sequencing and phylogenetic analysis

A comprehensive sequencing of the 16S rRNA gene spanning 1500 base pairs, encompassing both the reverse and forward reactions, was performed utilizing a genetic analyzer at the Central Dogma Lab, Invent Technologies Ltd., Dhaka. The sequencing process involved the utilization of both forward and reverse primers. Subsequently, the sequences underwent thorough editing to eliminate PCR primer binding sites and manual correction was carried out employing MEGA software. To ascertain the gene sequences' identity, they were automatically compared with bacterial sequences stored in databases (http://www.ncbi.nlm.nih.gov/) through BLAST analysis. A phylogenetic analysis was then constructed using the neighbor-joining algorithm.

Results

Colony forming unit (CFU)

The count of viable bacteria varied, ranging from 3.0×10^2 to 4.2×10^3 CFU/ml at a Tetracycline concentration of 32 µg/ml, and from 0.5×10^2 to 1.8×10^3 CFU/ml at a 64 µg/ml concentration (Table 2). The highest viable bacterial count (6.6×10^5 CFU/ml) was observed at the Rajshahi University-2 site (control), while the lowest count was recorded at the Durgapur site (64 µg/ml of TC) (Table 2). The prevalence of Tetracycline-resistant bacteria was noted to be between 0.27 to 1.28% for the 32 µg/mL concentration and 0.18 to 0.85% for the 64 µg/ml concentration, in relation to the total viable bacteria count.

Table 2: Viable count of TC resistant bacteria in fish farming pond water.

Sample sites	CFU/ml (Control)	Average CFU (CFU/ml) (Dose 32 µg/ml)	Average CFU (CFU/ml) (Dose 64 µg/ml)
Rajshahi University-1	4.7×10^5	6.0×10^2	4.0×10^2
Rajshahi University-2	6.6×10^5	1.8×10^3	1.2×10^3
Baghmara	2.6×10^5	1.5×10^3	1.3×10^3
Taherpur	3.4×10^5	1.1×10^3	7.0×10^2
Rajpara	3.3×10^5	2.3×10^3	1.5×10^3
Chapainawabgonj	6.3×10^5	2.0×10^3	3.0×10^2
Gomostapur	1.0×10^5	3.0×10^2	2.0×10^2
Kashiadanga	3.6×10^5	1.8×10^3	1.3×10^3
Tanore	6.3×10^5	4.2×10^3	1.8×10^3
Aradidhi	4.9×10^5	3.0×10^3	1.7×10^3
Durgapur	6.5×10^5	1.2×10^3	0.5×10^2
Puthia	6.8×10^5	3.8×10^3	2.5×10^2

Extraction of DNA and PCR amplification

An extensive study resulted in the retrieval of 12 distinct bacteria of six genera from water samples of twelve separate fish farming ponds. The use of agarose gel electrophoresis (Fig. 2) revealed various bacterial strains. Subsequent to isolating these strains, PCR products, each around 1500 base pairs, were directly sequenced. Notably, the sequencing outcomes demonstrated considerable homology, with similarities ranging from 98.43% to 99.88%, when compared with existing bacterial genera. Table 3 systematically displays the names of bacterial species, strain IDs, and their corresponding homology percentages.

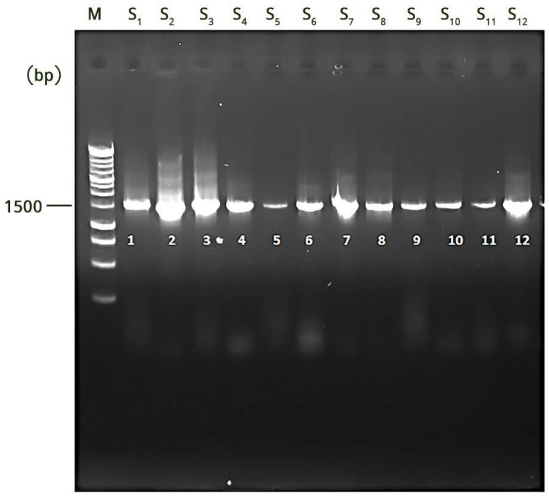

Fig. 2: Gel electrophoresis of 12 isolated samples.

Table 3: Molecular identification of resistant isolates.

Strain ID	Identified bacteria	Query coverage (%)
DUP-64	*Enterobacter asburiae* strain RA11	99.88
KAP-64	*Aeromonas* sp. strain J16OP4	100.00
BAP-32	*Acinetobacter* sp. EU18	99.54
RUP1-32	*Bacillus sp.* strain PYCC 8231	99.65
RAP-32	*Bacillus cereus* strain HAPH4	98.67
RAP-64	*Enterobacter cloacae* strain BY58	99.42
TAP-64	*Acinetobacter gandensis* strain ANC 4864	98.63
CHP-32	*Escherichia coli* strain ECIUP 24	99.65
CHP-64	*Shigella* sp. strain SCU-C1	100.00
ARP-64	*Enterobacter hormaechei* strain SU103	98.74
TAP-32	*Acinetobacter baumannii* strain S-X9A	99.35
TNP-32	*Bacillus altitudinis* strain AS_K1	98.76

The construction of phylogenetic tree (Fig. 3), facilitated by BLAST results, traced the evolutionary lineage of the bacteria by identifying related sequences across different species or organisms.

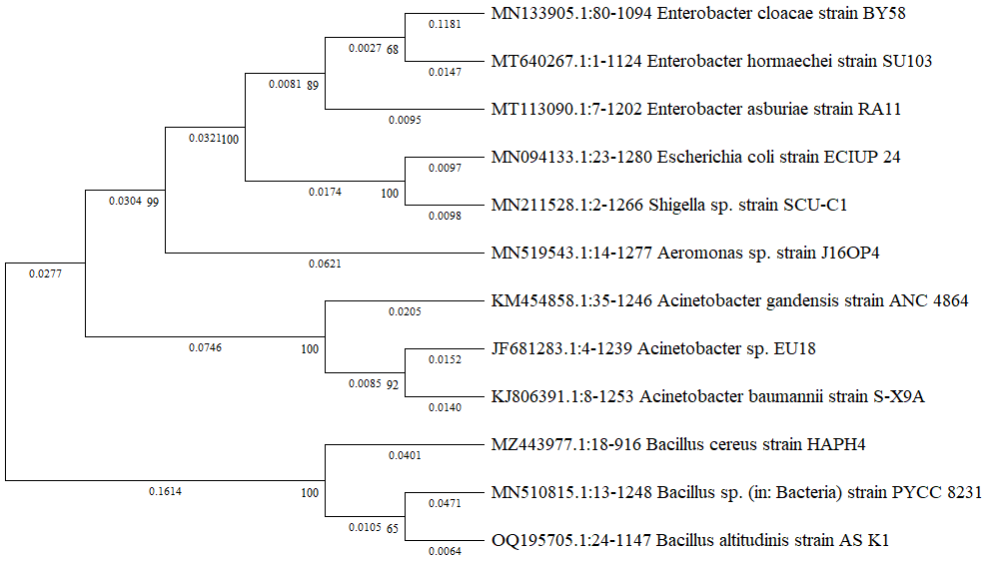

Fig. 3: Phylogenetic tree of 12 identified bacteria.

Discussion

Research into the bacterial composition in fish ponds contributes to informed decisions regarding appropriate management and strategies for disease control. This is particularly crucial as certain organisms within the bacterial flora may pose public health concerns. Reports highlight the emergence of antibiotic resistance as a substantial concern across various contexts, posing potential risks to human health. There is compelling evidence demonstrating the pivotal role of the aquatic environment in the spread of antibiotic-resistant bacteria and resistance genes throughout diverse ecosystems (Neela et al. 2015). Nevertheless, there is limited research on the surveillance of antibiotic resistance in the aquatic environments of Bangladesh. Antibiotic-resistant bacteria in fish ponds are influenced by factors like fish feeds, antibiotic use, water quality, and management practices. Studies show that extensive use of tetracycline in aquaculture fosters resistance (Sarmah et al. 2006). Poor water quality and inadequate waste management further promote resistant bacteria growth (Cabello 2006).

In our investigation, the number of viable bacteria fluctuated depending on the concentration of Tetracycline, with Rajshahi University-2 having the highest count and Durgapur the lowest. The prevalence of Tetracycline-resistant bacteria varied from 0.27% to 1.28% at 32 µg/mL and from 0.18% to 0.85% at 64 µg/ml. Comparable findings were presented by Neela et al. (2015) indicating a notable escalation in drug resistance levels among isolates from various manure samples. This underscores the potential of integrated poultry-fish farms as a contributing source to heightened antibiotic resistance. Abedin et al. (2020) conducted a study revealing a significant presence of antimicrobial-resistant bacteria and resistance determinants in fish ponds used for aquaculture in Bangladesh. Therefore, it can be inferred that the water in fish farming ponds plays a crucial role as a primary reservoir for hosting and disseminating drug-resistant bacteria within the aquatic ecosystem. In our research, we obtained 12 distinct bacteria belonging to six genera from water samples collected from twelve different fish farming ponds through 16S rRNA sequencing. Significantly, the sequencing results showed substantial similarity, ranging from 98.43% to 99.88%, when compared to known bacterial genera. The attributes of the microbial isolates from these pond water samples revealed the presence of twelve unique bacteria: *Enterobacter asburiae*, *Aeromonas* sp., *Acinetobacter* sp., *Bacillus* sp., *Bacillus cereus*, *Enterobacter cloacae*, *Acinetobacter gandensis*, *Escherichia coli*, *Shigella* sp., *Enterobacter hormaechei*, *Acinetobacter baumannii*, and *Bacillus altitudinis*. Our findings align with the research conducted by (Farzana et al. 2018), revealing the identification of five varieties of multidrug-resistant bacteria in fish samples obtained from the Noakhali Region in Bangladesh. Additionally, other studies have highlighted the widespread presence of tetracycline-resistant *E. coli* and *Salmonella* spp. in various water sources, including sewage, rivers, ponds, and swimming pools (Nahar et al. 2019). Moreover, research by (Sobur et al. 2019) has identified the presence of colistin-resistant *E. coli* in pond water, further emphasizing the issue of antibiotic resistance in aquatic environments.

The majority of bacteria identified in our study are pathogenic and can cause various diseases. *Escherichia coli* is notorious for causing serious foodborne illnesses, potentially leading to critical conditions like hemolytic uremic syndrome (Russo and Johnson 2003). *Shigella* sp. is responsible for shigellosis, characterized by severe, occasionally bloody diarrhea, posing significant risks especially to young children and those with weakened immune systems (Tosisa et al. 2020). *Acinetobacter baumannii* is a major concern in healthcare settings due to its role in causing various severe infections and its notable antibiotic resistance, making treatment challenging (Gedefie et al. 2021). *Bacillus cereus* is primarily associated with food poisoning, which can be intense, though typically not as life-threatening as infections caused by the aforementioned bacteria (Dietrich et al. 2021). Other bacteria such as *Enterobacter*, *Aeromonas*, and various

Acinetobacter species also pose risks, especially in immunocompromised individuals or within hospital environments where infections are harder to manage. In contrast, *Bacillus altitudinis* and other *Bacillus* species, apart from *B. cereus*, are generally less implicated in causing severe human diseases. Tetracycline-resistant bacteria in fish ponds pose significant risks to public health and aquaculture. They can enter the human food supply through contaminated fish, causing health issues and reducing treatment effectiveness (Martinez 2009). Furthermore, the spread of resistance genes in water can undermine antibiotics used in human and veterinary medicine (Kümmerer 2009).

Conclusion

In summary, our comprehensive study reveals that in pond water from fish farms in Rajshahi region, Bangladesh, viable bacteria ranged from 4.2×10^3 to 3.0×10^2 CFU/ml at 32 µg/ml Tetracycline, and from 1.8×10^3 to 0.5×10^2 CFU/ml at 64 µg/ml. The prevalence of Tetracycline-resistant bacteria was between 0.27% and 1.28%. This indicates that even low concentrations of the antibiotic in pond water can foster resistance, potentially leading to the spread of antibiotic resistance bacteria and posing a risk to public health through the consumption of fish from these ponds. Through 16S rRNA sequencing, we identified 12 bacteria across six genera, with some closely matching (98.43% to 99.88%) known pathogenic strains. These results highlight the importance of implementing preventive measures against Tetracycline-resistant bacteria in fish farms and conducting further research to identify their sources and transmission routes to human health.

Acknowledgements

All praise to the Almighty Allah. I would like to extend my sincere thanks to my respected supervisor Dr. Md. Habibur Rahman, Professor Dept. of Zoology, University of Rajshahi. I am grateful to Dept. of Zoology & Dept. of Botany (Environmental Microbiology Lab.), University of Rajshahi for giving laboratory facilities. I am also grateful to IBSc, University of Rajshahi for providing Fellowship.

Conflict of interest

The authors assert that they have no conflicts of interest associated with the publication of this article.

References

Abe K, Nomura N and Suzuki S (2020). Biofilms: Hot spots of horizontal gene transfer (HGT) in aquatic environments, with a focus on a new HGT mechanism. FEMS Microbiology Ecology 96 (5): 1-12.

Abedin MZ, Rahman MS, Hasan R, Shathi JH, Jarin L and Zaman MS (2020). Isolation, Identification, and Antimicrobial Profiling of Bacteria from Aquaculture Fishes in Pond Water of Bangladesh. American Journal of Pure and Applied Biosciences 2(3): 39-50.

Asif AAl, Hossain A, Hamli H, Islam S and Kabir SL (2021). Research trends of aqua medicines, drugs and chemicals (AMDC) in Bangladesh: the last decade's (2011-2020) story to tell. Asian Journal of Medical and Biological Research 7: 90-106.

Bondad-Reantaso MG, MacKinnon B, Karunasagar I, Fridman S, Alday-Sanz V, Brun E, Le Groumellec M, Li A, Surachetpong W, Karunasagar I, Hao B, Dall'Occo A, Urbani R and Caputo A (2023). Review of alternatives to antibiotic use in aquaculture. Reviews in Aquaculture 15: 1421-1451.

Brooks BD and Brooks AE (2014). Therapeutic strategies to combat antibiotic resistance. Advanced Drug Delivery Reviews 78: 14-27.

Cabello FC (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environmental Microbiology 8(7): 1137-1144.

Dewi RR, Hassan L, Daud HM, Matori MF, Zakaria Z, Ahmad NI, Aziz SA and Jajere SM (2022). On-Farm Practices associated with multi-drug-resistant *Escherichia coli* and *Vibrio parahaemolyticus* derived from cultured fish. Microorganisms 10(8): 1520.

Dey MM, Bose ML and Alam MF (2008). Country case study: development and status of freshwater aquaculture in Bangladesh. World Fish Centre Studies and Reviews No.1872. The World Fish Centre, Penang, Malaysia. p. 73.

Dietrich R, Jessberger N, Ehling-Schulz M, Märtlbauer E and Granum PE (2021). The food poisoning toxins of *Bacillus cereus*. Toxins 13(2): 98.

Gedefie A, Demsis W, Ashagrie M, Kassa Y, Tesfaye M, Tilahun M, Bisetegn H and Sahle Z (2021). Acinetobacter baumannii biofilm formation and its role in disease pathogenesis: A review. Infection and Drug Resistance 14: 3711-3719.

Hossain FE, Chakraborty S, Bhowmick NC and Rahman MA (2018). Comparative analysis of antibiotic resistance pattern of bacteria isolated from fish of cultured and natural ponds: a study based on Noakhali region of Bangladesh. Bioresearch Communications 4: 586–591.

Huang Y, Zhang L, Tiu L and Wang HH (2015). Characterization of antibiotic resistance in commensal bacteria from an aquaculture ecosystem. Frontiers in Microbiology 6: 153905.

James G (2010). Universal bacterial identification by PCR and DNA sequencing of 16S rRNA gene. PCR for clinical microbiology: an Australian and international perspective. 1499 Sutherland, NSW, Australia, pp. 209-214.

Kümmerer K (2009). Antibiotics in the aquatic environment–a review–part I. Chemosphere 75(4): 417-434.

Martinez JL (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. Environmental Pollution 157(11): 2893-2902.

Miranda CD and Zemelman R (2002). Bacterial resistance to oxytetracycline in Chilean salmon farming. Aquaculture 212: 31-47.

Mortuza MG and Hossain MA (2006). The fisheries resources management of the greater Rajshahi District, Bangladesh. University Journal of Zoology, Rajshahi University 25: 67-8.

Nahar A, Islam MA, Sobur MA, Hossain MJ, Zaman SB, Rahman MB and Rahman MT (2019). Detection of tetracycline resistant *E. coli* and *Salmonella spp*. in sewage, river, pond and swimming pool in Mymensingh, Bangladesh. African Journal of Microbiology Research 13(25): 382-387.

Neela FA, Banu MSTNA, Rahman MA, Alam MF and Rahman MH (2015). Occurrence of antibiotic resistant bacteria in pond water associated with integrated poultry-fish farming in Bangladesh. Sains Malaysiana 44: 371-377.

Reasoner DJ (2004). Heterotrophic plate count methodology in the United States. International Journal of Food Microbiology 92: 307–315.

Russo TA and Johnson JR (2003). Medical and economic impact of extraintestinal infections due to *Escherichia coli*: Focus on an increasingly important endemic problem. Microbes and Infection 5: 449-456.

Salma U, Shafiujjaman M, Al Zahid M, Faruque MH, Habibullah-Al-Mamun M and Hossain A (2022). Widespread use of antibiotics, pesticides, and other aqua-chemicals in finfish aquaculture in Rajshahi District of Bangladesh. Sustainability 14(24): 17038.

Sarmah AK, Meyer MT and Boxall AB (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65(5): 725-759.

Sobur MA, Ievy S, Haque ZF, Nahar A, Zaman SB and Rahman MT (2019). Emergence of colistin-resistant *Escherichia coli* in poultry, house flies, and pond water in Mymensingh, Bangladesh. Journal of Advanced Veterinary and Animal Research 6: 50-53.

Tosisa W, Mihret A, Ararsa A, Eguale T and Abebe T (2020). Prevalence and antimicrobial susceptibility of *Salmonella* and *Shigella* species isolated from diarrheic children in Ambo town. BMC Pediatrics 20: 1-8.

Wright MH, Adelskov J and Greene AC (2017). Bacterial DNA extraction using individual enzymes and phenol/chloroform separation. Journal of Microbiology and Biology Education 18(2): 10-128.

(Manuscript received on 25th March 2024 and revised on 29th April 2024)