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Preliminary survey of biofilm forming, antibiotic resistant *Escherichia coli* in fishes from land based aquaculture systems and open water bodies in Bangladesh

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The emergence and spread of multidrug-resistant pathogens, such as *Escherichia coli*, present major global public health challenges. This study aimed to investigate the prevalence, antibiotic resistance patterns, biofilm production, and the presence of antibiotic resistance genes (ARGs) and biofilm-forming genes in *E. coli* isolated from fish in open-body water (wild) sources and land-based aquaculture (cultured) systems in Mymensingh, Bangladesh. We collected 130 fish (Koi: *Anabas testudineus* and Shing: *Heteropneustes fossilis*) among which 70 were from wild sources and 60 from cultured systems. We screened 116 probable *E. coli* isolates through selective culture, Gram-staining, and biochemical tests. Using *malB* gene-specific PCR, we confirmed 87 isolates (67.0%) as *E. coli*. Cultured fish had a higher prevalence (70.0%) compared to wild fish (64.0%). Biofilm formation was detected in 20.0% *E. coli* by Congo red agar tests. However, crystal violet assays revealed that 70.0% of *E. coli* from cultured fish produced biofilm, compared to 20.0% from wild fish, with 7.0% of cultured fish isolates showing strong biofilm production. Antibiotic resistance profiling showed that 100.0% *E. coli* isolates were resistant to ampicillin and ceftazidime, beta-lactamase-producing antibiotics. Resistance patterns varied by source, with nearly 97.0% of *E. coli* from cultured fish being multidrug-resistant (MDR), compared to 60.0% in wild fish. *E. coli* from cultured fish were identified as potential reservoirs of ARGs such as *blaTEM* (83.0%), *blaSHV* (81.0%), *blaCTX* (78.57%), and the biofilm forming gene *fimC* (100.0%). Significant associations were observed for *blaTEM* ($p = 0.033$), *blaSHV* ($p = 0.038$), and *fimC* ($p = 0.005$). These findings highlight the need for monitoring β-lactamase-resistant and biofilm-forming *E. coli* in both wild and cultured fish in Bangladesh due to their potential threat to public health and animal populations.

Keywords *E.coli*, Biofilm production, Antibiotic resistance, Wild and cultured fish, Public health, Bangladesh

*Escherichia coli*is an exceptionally adaptable microorganism, capable of thriving in a wide range of environmental settings[1,2]. Its diverse characteristics have made it the most extensively studied microorganism in the world[3]. *E. coli*is a natural inhabitant of the gastrointestinal tract in humans and animals[4–6], where it plays a crucial role in maintaining gut health and digestion. Fecal microorganisms have the potential to endure prolonged periods in soils and manure and water and therefore, they serve as an accessible source of contamination[7]. It is also found in wastewater, where it can persist under varying conditions, often serving as an indicator of fecal contamination[8]. Its versatility allows it to survive and multiply in diverse environments, ranging from nutrient-rich to nutrient-

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poor conditions, making it one of the most studied and significant bacteria in both environmental and medical microbiology[1,2].

Antimicrobial resistance (AMR) is one of the most significant threats to global health in the twenty-first century[9]. AMR occurs when microorganisms develop resistance to antibiotics designed to combat them[10]. This resistance complicates infection treatment due to a lack of effective therapeutics and can lead to multidrug resistance (MDR), posing future challenges in managing infectious diseases[11]. Overuse of broad-spectrum and unnecessary antibiotics, often without medical guidance, exacerbates resistance issues[11,12]. The World Health Organization predicts that by 2050, 10 million deaths annually will result from MDR bacteria[13]. The extensive use of antibiotics in livestock, agriculture, aquaculture, and healthcare sectors promotes the emergence of antibiotic-resistant strains in aquaculture and open-water sources[14]. Recently, *E. coli*has shown a growing resistance to many commonly used antibiotics[15]. *E. coli*is becoming increasingly difficult to treat due to its evolving resistance to most first-line antimicrobials[16]. Additionally, resistant *E. coli* strains can transfer antibiotic resistance genes to other *E. coli*strains and bacteria within the gastrointestinal tract, leading to the acquisition of resistance from external organisms[17]. The global prevalence of MDR *E. coli*in both humans and animals is on the rise[2,18,19]. The growing resistance of *E. coli*to beta-lactam antibiotics is causing significant challenges in the general population[20]. Moreover, *E. coli*can develop resistance to various classes of commonly prescribed antibiotics, such as trimethoprim-sulfamethoxazole, aminoglycosides, and fluoroquinolones[21]. Some *E. coli*strains produce Extended Spectrum Beta-lactamase (ESBL) enzymes, which can interact with and break down the active compounds in various commonly used antibiotics, rendering them ineffective[22,23]. Approximately 10% of all *E. coli*strains isolated worldwide are ESBL-producers[22]. Another factor contributing to development of MDR in *E. coli*is its ability to produce biofilms. Biofilms serve as a protective mechanism for bacteria, allowing them to survive in harsh conditions and communicate with one another, forming a collective resistance against antibiotics[24]. Bacteria adhere to surfaces and secrete Extracellular Polymeric Substances (EPS) to form a protective shell. This biofilm structure makes it difficult for antibiotics to penetrate and effectively kill the bacteria[25].

Bangladesh, a Southeast Asian country with the highest number of natural water bodies, is home to thousands of fish species[26]. Fish is a crucial component of Bangladeshi cuisine and culture. In response to the increasing market demand, fish cultivation has expanded through hatcheries and bio-flock systems[27]. This growth involves many people working in and around the fish industry. Hazardous microorganisms present in these fish pose a potential risk to local populations, particularly those handling, cutting, and cooking fish in the market[28]. The presence of antibiotic-resistant *E. coli*in fish not only poses a direct threat to human health through foodborne illnesses but also contributes to the global spread of resistance genes, complicating treatment options for infections[29]. In aquaculture, the persistence of resistant bacteria, enhanced by biofilm formation, challenges the industry's sustainability, leading to increased disease management costs and potential economic losses[30]. Moreover, the discharge of these resistant strains into natural water sources disrupts aquatic ecosystems, further exacerbating environmental degradation[30]. The presence of MDR *E. coli* in fish represents a significant global health threat. Despite extensive research on *E. coli* in commercially farmed fish, there remains a considerable knowledge gap regarding its occurrence in fish from open-body water sources and land-based aquaculture systems in Bangladesh. Furthermore, currently there is a paucity of data concerning MDR *E. coli* in these environments. This study therefore investigated the prevalence, antibiotic resistance patterns, biofilm production, presence of ARGs, and biofilm forming gene in *E. coli* isolated from fish populations belonged to open-body water (wild) and land-based aquaculture (cultured) systems in Mymensingh district of Bangladesh, employing both phenotypic and genotypic techniques.

Results

Overall prevalence of *E. Coli* in wild and cultured fish populations

Out of 130 fish samples, which comprised specimens from both wild sources ($n=70$) and cultured systems ($n=60$), the overall of prevalence of *E. coli* was 66.92% (87/130). Specifically, *E. coli* was detected in approximately 64% (45/70) of the wild fish samples (95% CI: 53–74%) and 70.0% (42/60) of the cultured fish samples (95% CI: 57–80%) (Fig. 1). This analysis reveals a significant prevalence of *E. coli* in both wild and cultured fish populations. However, bivariate analysis did not detect a significant correlation ($p>0.5$) in the prevalence of *E. coli* between these two types of samples.

Biofilm formation capabilities of the *E. Coli*

The Congo Red Agar (CRA) plate test showed low positivity in the presumptive detection of biofilm formation in 20.0% of the analyzed *E. coli* isolates. Quantitative biofilm formation by *E. coli* isolates was assessed using a crystal violet assay. Biofilm quantification analyses showed that 80.0% of the *E. coli* isolates from wild sources did not exhibit any biofilm formation (Fig. 2). Conversely, 70.0% of the *E. coli* isolates from cultured systems were biofilm producers, indicating that this technique was more efficient than CRA for the detection of biofilm production. Our analysis also revealed that about 20% of the *E. coli* isolates from both types of samples exhibited intermediate-level biofilm production (Fig. 2). Furthermore, 7% of the isolates from cultured systems were classified as strong biofilm producers, whereas none of the isolates from wild fishes demonstrated strong biofilm production (Fig. 2).

Antibiotic resistance profile of the *E. Coli*

The overall results of the Kirby-Bauer disc diffusion method for antibiotic susceptibility testing are illustrated in Fig. 3. Both types of isolates, those from wild source and those from cultured systems, demonstrated significant resistance to various antibiotics (**Table S1, and Table S2**). Specifically, all isolates from both sample types exhibited 100.0% resistance (95% CI: 95.77–100%) to ampicillin and ceftazidime. Resistance patterns varied

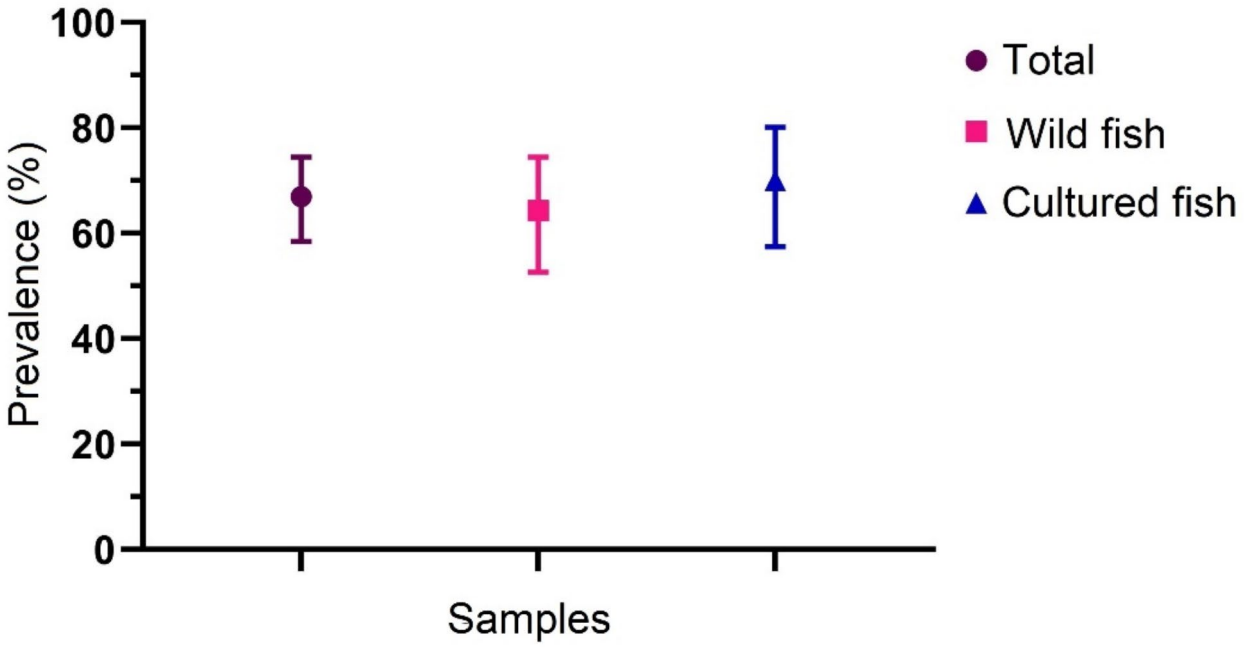

Fig. 1. Prevalence of *E. coli* isolated from fish in open-body water (wild fish) and land-based (cultured fish) aquaculture systems in Mymensingh, Bangladesh.

for other antibiotics among the isolates from both sources. In isolates from wild fish, approximately 33.0% exhibited resistance to imipenem, while about 66.0% demonstrated intermediate-resistance to ciprofloxacin (Fig. 3a). Bivariate analysis revealed a highly significant positive correlation between ceftriaxone and gentamicin ($\rho = 1.000$; $p = 0.000$). Conversely, negative correlations were observed between imipenem and tetracycline ($\rho = -0.303$; $p = 0.043$), ceftriaxone and imipenem ($\rho = -0.303$; $p = 0.043$), and gentamicin and imipenem ($\rho = -0.303$; $p = 0.043$) (**Table S3**). Additionally, approximately 60.0% of the *E. coli* isolates from wild sources were classified as MDR, exhibiting resistance to three or more antibiotics from different classes. These isolates showed four distinct MDR patterns, with a multiple antibiotic resistance (MAR) index of 0.2 to 0.4 (Table 1). In addition, approximately 45.0% of these isolates (95% CI: 31.22–60.05%) showed resistance to imipenem (Fig. 3b). Furthermore, around one-third (29.0%) of the isolates from cultured systems demonstrated resistance to both ceftriaxone and fosfomycin. Bivariate analysis revealed a significant positive correlation between ceftriaxone and ciprofloxacin ($\rho = 0.615$; $p = 0.000$). Conversely, negative correlations were observed between imipenem and ciprofloxacin ($\rho = -0.358$; $p = 0.020$), gentamicin and ceftazidime ($\rho = -0.343$; $p = 0.026$), gentamicin and fosfomycin ($\rho = -0.325$; $p = 0.036$), and gentamicin and imipenem ($\rho = -0.441$; $p = 0.003$) (**Table S4**). Isolates from fishes belonged to cultured systems exhibited five distinct MDR patterns, with a MAR index ranging from 0.2 to 0.5 (Table 1). Nearly all of these isolates (97.0%) were classified as MDR (95% CI: 87.67–99.87%), as illustrated in Fig. 3c.

Antibiotic resistance and biofilm forming genes in *E. Coli*

The profiles of antibiotic resistance and biofilm forming genes in *E. coli* isolates ($N = 87$) were analyzed to assess the potential risks these strains pose to public health and food safety (Fig. 4a). Using gene-specific polymerase chain reaction (PCR) (**Table S5**), we analyzed 45 *E. coli* isolates from fish populations of wild origin for determining the resistance and biofilm production gene profiles (Fig. 4b). PCR results showed that 28 isolates (66.66%, 95% CI: 51.55–78.98%) tested positive for the *blaTEM* gene, associated with beta-lactamase production. Similarly, 27 isolates (61.36%, 95% CI: 46.66–74.27%) were positive for the *blaSHV* gene, another beta-lactamase marker (Fig. 4b). The *blaCTX* gene, linked to extended-spectrum beta-lactamase production, was detected in 28 isolates (66.66%, 95% CI: 51.55–78.98%). Resistance associated with the *sul1* gene was found in 20 isolates (47.61%, 95% CI: 33.36–62.27%). Lastly, the biofilm forming gene *fimC*, associated with fimbrial adhesins, was detected in 40 isolates (95.23%, 95% CI: 84.21–99.15%) (Fig. 4b).

Similarly, gene-specific PCR (**Table S5**) was employed to determine the resistance and biofilm production gene profiles in 42 *E. coli* isolates from fish in cultured systems (Fig. 4c). Our results showed that 35 of these isolates (83.33%, 95% CI: 69.39–91.68%) harbored the *blaTEM* gene, known for producing beta-lactamase enzyme that helps bacteria resist certain antibiotics. Additionally, 34 isolates (80.95%, 95% CI: 66.69–90.01%) tested positive for the *blaSHV* gene, another beta-lactamase marker gene. The *blaCTX* gene was found in 33 isolates (78.57%, 95% CI: 64.04–88.29%). Moreover, 22 isolates of *E. coli* were found to harbor *sul1* (52.38%, 95% CI: 37.72–66.64%) gene. Remarkably, all *E. coli* isolates (100.0%, 95% CI: 91.62–100.00%) from cultured fish populations were found to harbor the *fimC* gene (Fig. 4c). This gene is crucial for the production of fimbrial

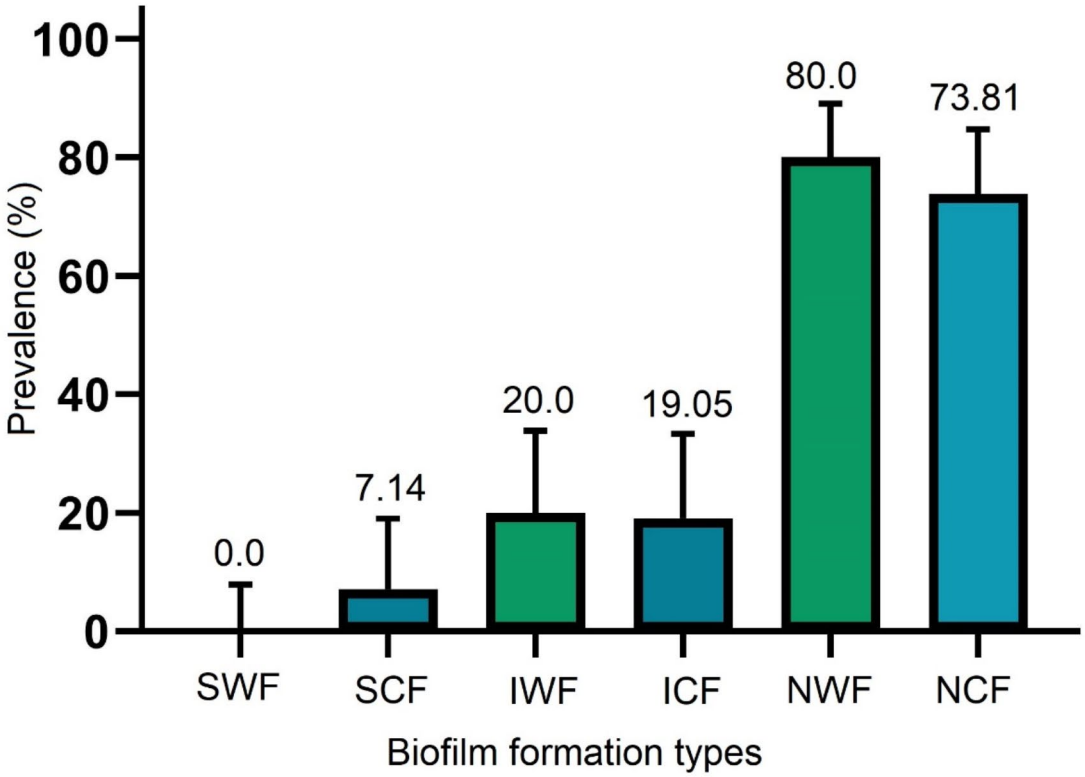

Fig. 2. Prevalence of biofilm forming *E. coli* isolated from fish in open-body water (wild fish) and land-based (cultured fish) aquaculture systems. Here, SWF = strong biofilm producer in wild fish, SCF = strong biofilm producer in cultured fish, IWF = intermediate biofilm producer in wild fish, ICF = intermediate biofilm producer in cultured fish, NWF = non-biofilm producer in wild fish, and NCF = non-biofilm producer in cultured fish.

adhesins, which facilitate bacterial adherence to surfaces, thereby indicating a high potential for biofilm formation among these isolates. Furthermore, the bivariate analysis revealed a significant association between the presence of the *blaTEM* gene ($p = 0.033$), the *blaSHV* gene ($p = 0.038$), and the biofilm forming gene *fimC* ($p = 0.005$) in *E. coli* isolates from fishes belonged to both wild source and cultured aquaculture systems.

Discussion

Bangladesh, the largest delta of the world, is covered with water for much of the year. Fish play a crucial role in the economy and cuisine of Bangladesh. While previous studies have investigated pathogenic bacteria in fish from Bangladesh[31–33], there is no published data on the comparative analysis of antibiotic-resistant *E. coli* and their biofilm formation abilities in fish from closed and open water sources. To address this gap, this study investigated the prevalence, antibiotic resistance profiles, biofilm formation, the presence of ARGs, and virulence genes in *E. coli* isolates circulating in fish populations sourced from open-body water (wild) and land-based aquaculture (cultured) systems in Mymensingh, Bangladesh, utilizing both phenotypic and genotypic methods. We analyzed 130 fish samples from wild source and cultured systems. Our study revealed that *E. coli* was present in approximately 67.0% of the tested samples, with a notably higher prevalence in fish from cultured systems compared to those from wild sources. Specifically, *E. coli*was detected in 70% of fish collected from cultured systems and 64% of fish from wild sources. In Bangladesh, the majority of fish farms are located in densely populated areas, where farming practices often include the use of poultry droppings as a feed source for farmed fish[34]. This practice likely contributes to the elevated levels of *E. coli* detected in samples collected from these farm environments. Supporting this investigation, a study by Ava et al. (2020) reported 75% prevalence of *E. coli*in fish from the Dinajpur district of Bangladesh, a finding that closely mirrors our results[35]. This finding suggests that the conditions and practices within these farming setups may play a significant role in the contamination of fish with *E. coli*. Additionally, a similar prevalence of *E. coli*was reported in shrimp samples in Iraq[36], indicating that the issue of *E. coli* contamination in aquaculture may be widespread, influenced by local farming practices and environmental conditions.

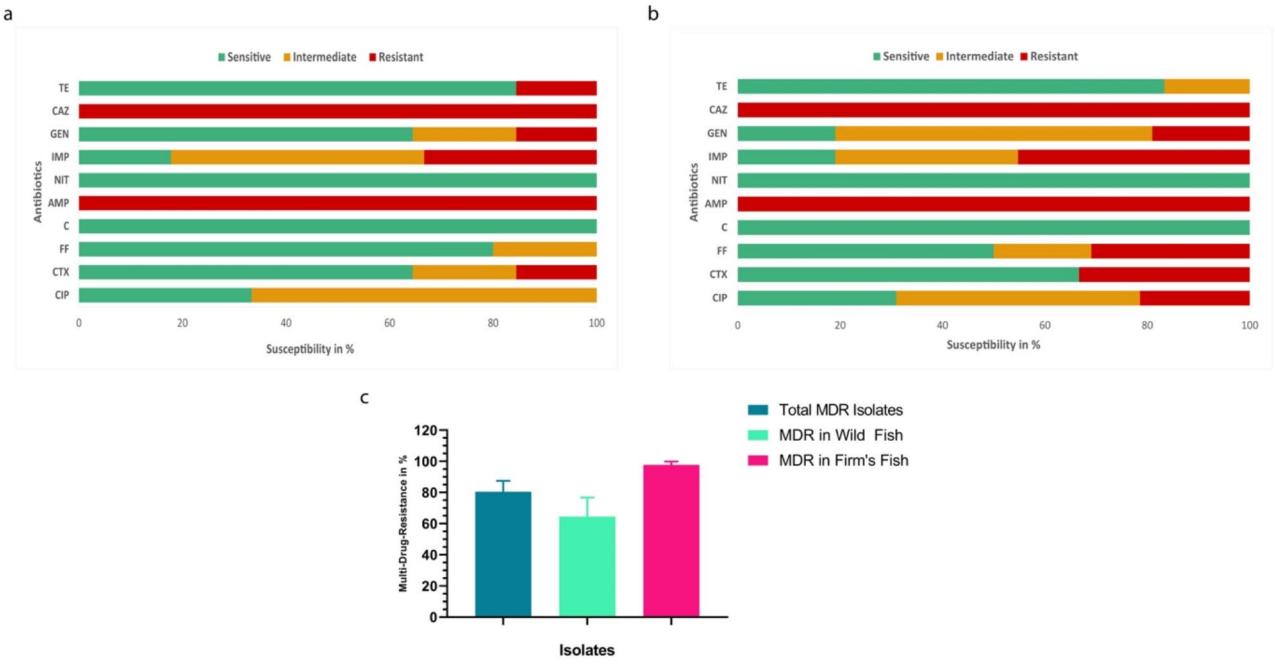

Fig. 3. Antibiotic resistance in *E. coli* isolates. (a) Antibiotic resistance in *E. coli* isolates from open body water (wild fish) sources. (b) Antibiotic resistance in *E. coli* isolates from land-based (cultured fish) aquaculture systems. (c) Multidrug resistance (MDR) patterns in in *E. coli* isolates sourced from wild fish and cultured fish. Here, TE = Tetracycline, CAZ = Ceftadizime, GEN = Gentamycin, IMP = Imipenem, NIT = Nitrofurantoin, AMP = Ampicillin, C = Chloramphenicol, FF = Fosfomycin, CTX = Ceftriaxone, CIP = Ciprofloxacin.

Resistance patterns	Number of isolates	Number of resistant antibiotics in different classes	MDR (%)	MAR index
E. coli isolates from wild fish				
AMP, CAZ	16	2(2)	29/45 (64.44)	0.2
AMP, CAZ, TE	7	3(3)		0.3
AMP, IMP, CAZ	15	3(3)		0.3
CTX, AMP, GEN, CAZ	7	4(4)		0.4
E. coli isolates from cultured fish				
AMP, CAZ	1	2(2)	41/42 (97.61)	0.2
AMP, GEN, CAZ	20	3(3)		0.3
FF, AMP, CAZ	7	3(3)		0.3
CIP, AMP, IMP, CAZ	8	4(4)		0.4
CTX, FF, AMP, IMP, CAZ	6	5(5)		0.5

Table 1. Multidrug-resistance patterns of *E. Coli* isolates from open body water (wild fish, no human involvement during raising) sources and land-based aquaculture (cultured fish, cultivated by humans for business purposes in hatchery of ponds) systems.

The quantitative crystal violet microtiter plate (CVMT) tests provided insightful results regarding biofilm formation in *E. coli* isolates. Specifically, 7% of *E. coli* isolates from cultured environments were identified as strong biofilm producers, indicating a significant ability to form biofilms in these controlled settings. In contrast, no strong biofilm producers were detected in the isolates from wild sources, suggesting a lower prevalence of robust biofilm formation in these more variable natural environments. These findings contrast with a previous study by Onmaz et al., who investigated reported 24.0% *E. coli*isolates from fish samples obtained from different markets in Turkey were strong biofilm producers[37], highlighting a higher rate of strong biofilm formation compared to our findings. In a recent study from Bangladesh, researchers investigated *E. coli*in beef and found that 19.0% of their isolates were strong biofilm producers[38]. While these results indicate a higher prevalence of strong biofilm-producing *E. coli* compared to our findings, it is important to consider that this difference may be due to variations in sampling locations and sample types. This discrepancy may be attributed to differences

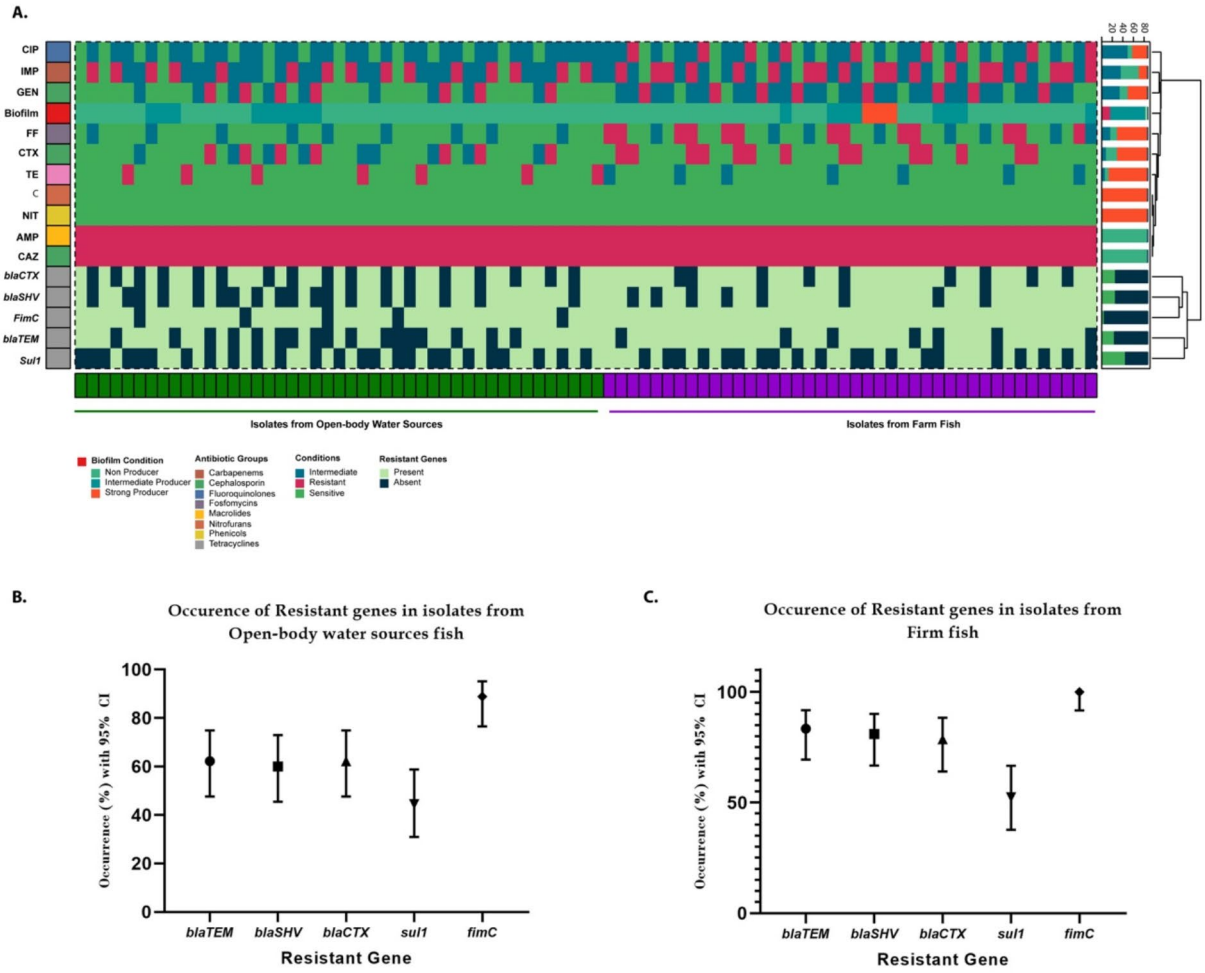

Fig. 4. Prevalence of antibiotic resistance genes (ARGs; *blaTEM*, *blaSHV*, *blaCTX* and *sul1*)) and biofilm forming gene (*fimC*) in *E. coli* isolated from fish in open-body water (wild fish) and land-based (cultured fish) aquaculture systems. (a) Representative heatmap showing the prevalence of *E. coli* isolates resistant to different antibiotics and antibiotic-resistant genes. Here, TE = Tetracycline, CAZ = Ceftadizime, GEN = Gentamycin, IMP = Imipenem, NIT = Nitrofurantoin, AMP = Ampicillin, C = Chloramphenicol, FF = Fosfomycin, CTX = Ceftriaxone, and CIP = Ciprofloxacin. (b) Prevalence of ARGs and biofilm forming gene in *E. coli* isolated from wild fish, and (c) Prevalence of ARGs and biofilm forming gene in *E. coli* isolated from cultured fish.

in environmental conditions, aquaculture practices, or geographical variations, underscoring the need for localized studies to understand the factors influencing biofilm formation in *E. coli* across different settings. In addition, it observed variation in the biofilm-forming ability of the isolated *E. coli* might be under the influence of methodologies used here e.g., Congo Red vs. Crystal Violet assay.

The examination of *E. coli* isolates from fish populations in both wild and cultured sources revealed the presence of various antibiotic-resistant phenotypes. In this study, we evaluated the resistance profiles of *E. coli*isolates from both wild and cultured fishes against the 10 most commonly used antibiotics in Bangladesh. In this study we detected 66.0% isolates of wild fish as intermediate-resistance to ciprofloxacin. While in a previous study in the same type of fish from Mymensingh, all isolates were found to be sensitive to ciprofloxacin[31]. This observed variation could be linked to temporal variation. In addition, our findings indicated that all isolates from both types of fish samples were resistant to ampicillin and ceftazidime. While no fosfomycin-resistant *E. coli*was found in wild fish, whereas approximately 30.0% of the isolates from cultured fish were resistant to fosfomycin. This profile of AMR may be attributed to the frequent use of antibiotics in fish farming for therapeutic purposes and growth promotion[39]. The high resistance to ampicillin and ceftazidime is particularly alarming, as these critical beta-lactam antibiotics are commonly used in both livestock, including fish, and human medicine[40,41]. The detection of antibiotic-resistant *E. coli*in the studied samples suggests that these fish may serve as spreaders of resistant microorganisms throughout aquatic environments[42]. Furthermore, 97.0% of isolates from cultured fish exhibited MDR, while approximately 60.0% of isolates from wild fish demonstrated MDR patterns. The widespread and often unregulated use of antibiotics without proper medical prescriptions has led to a concerning

increase in antimicrobial resistance among microorganisms in Bangladesh and other Southeast Asian countries in recent years[43]. A key finding of this study is the investigation of the five most prominent ARGs in *E. coli*. This information is crucial for developing a surveillance program to help control and reduce the spread of ARGs in the environment. We found that the *blaTEM*, *blaSHV*, and *blaCTX* genes were more frequently present in *E. coli* isolates from cultured fish, with occurrences ranging from 78.0 to 83.0%. In contrast, these genes were less common in isolates from wild fish populations, where their prevalence ranged from 61.0 to 66.0%. This finding suggests a higher level of antibiotic resistance in cultured fishes, potentially linked to the practices and conditions in cultured aquaculture environments. A previous study conducted by Bora et al. in India reported 78.0% and 89.0% prevalence of the *blaTEM* and *blaCTX*genes[44] in *E. coli*, a finding that aligns closely with our results. This similarity underscores a significant and consistent occurrence of the *blaTEM* and *blaCTX* genes across different regions, highlighting its widespread presence in antibiotic-resistant *E. coli* populations. Another survey conducted by Goudarzi et al. reported a 69% prevalence of the *blaTEM*gene in their investigation[45]. This finding is somewhat lower compared to the higher prevalence observed in our study, where *blaTEM* was found in 78.0–83.0% of the isolates from cultured fish. This discrepancy highlights variations in resistance gene prevalence across different regions and studies, suggesting that local factors may influence the distribution of antibiotic-resistant genes. Such findings emphasize the need for continued monitoring and control measures to address the spread of this resistance gene in both local and broader contexts. Additionally, to identify virulence gene in *E. coli*, we examined the samples for the biofilm forming gene *fimC*, which facilitates adhesion of uropathogenic *E. coli*(UPEC) to epithelial cells during infections[46]. The biofilm forming gene *fimC* was present in all (100%) of the fish samples from cultured environments, whereas its prevalence was lower (95.23%) in fish samples from wild sources. Although there is limited public data on the *fimC* gene in *E. coli* from fish, a study by Islam et al. reported a 67.0% occurrence of the *fimC* gene in *E. coli*isolated from migratory birds in Bangladesh[47]. It is also important to note that in this study we found inconsistency between the *fimC* genotype of *E. coli* and its phenotypic ability to form biofilm. Biofilm formation is a complex mechanism and there are many other genes in addition to *fimC* involved in the process. Observed inconsistency may be due to involvement of other genes in *E. coli* biofilm formation.

Important limitation of this study is the lack of more samples, however, we designed this study to generating base line data, since no such study was earlier carried out in these fishes in Bangladesh. Koi and Shing are different species of fish. They have different feeding behavior and natural niche adaptation. It is therefore not also unlikely to have variation in load and character of *E. coli* harbor in these fishes. Many of the variation observed in biofilm formation and AMR in *E. coli* isolated from wild and cultured environment might also be linked with this variation.

Conclusion

This study sought to compare the prevalence, antibiotic resistance, biofilm formation, antibiotic resistance and virulence gene profiles in *E. coli* isolated from fish populations in wild source and cultured aquaculture systems in Bangladesh, using both phenotypic and genotypic methods. The overall prevalence of *E. coli* was about 67.0%. The biofilm assay identified 20.0% of *E. coli* from both sources as positive for biofilm formation. Isolated *E. coli* showed varying resistance patterns and significant correlations between different antibiotics. MDR *E. coli* isolates were more prevalent in fish populations from cultured aquaculture systems compared to those from fish populations of wild sources. A significant association was found between *blaTEM*, *blaSHV*, and biofilm forming gene *fimC*. The potential horizontal transfer of these ARGs to other pathogens in both wild sources and cultured aquaculture systems poses serious public health risks. The emergence and spread of MDR *E. coli* in these environments underscore the urgency for ongoing surveillance and intervention.

Materials and methods

Sample collection and processing

This cross-sectional study was undertaken at the Bacteriology Laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh, Bangladesh. The studied samples were sourced from multiple markets across the Mymensingh district of Bangladesh (**Fig. S1**). We collected a total of 130 Koi (*A. testudineus;* size = ± 5 cm, weight = ± 20 g) and Shing (*H. fossilis;*size = ± 10 cm, weight = ± 18 g) fish. Shing and Koi are the two most abundant types of fish found in local markets and people take them daily as their sources of protein in Bangladesh. Fishermen and consumers frequently encounter these fish and there is a high chance of getting affected by the pathogenic bacteria in those fish. Since these fish are most common and popular in mass people, we selected them as sample for our current study. Among them 70 fish were from open body water sources (40 were Koi fish and 30 were Shing fish) and 60 from culture systems (30 were Koi and 30 were Shing). The samples were immediately transported to the Laboratory, maintaining a cold chain at 4 °C. Upon arrival at the laboratory, the samples were processed under aseptic conditions[48]. Approximately, 1 g portion from intestine of each fish sample was placed in a mortar and pestle, and ground with Phosphate Buffered Saline (PBS). Subsequently, 1 mL of the homogenized sample was transferred into a 30 mL test tube containing 9 mL of nutrient broth. The tubes were incubated overnight at 37 °C to enrich the target bacteria[48,49].

Isolation and identification of *E. Coli*

To isolate *E. coli*, following overnight enrichment, a loopful (~ 10 µL) of the enriched specimen was aseptically streaked onto Eosin-Methylene Blue (EMB) agar plates (HiMedia, Mumbai, Maharashtra, India) using a sterilized inoculation loop. The plates were then incubated overnight at 37 °C. Colonies displaying a characteristic metallic sheen were presumptively identified as *E. coli*[18,50]. These presumptive colonies were subsequently subcultured by transferring them to fresh EMB agar plates to obtain isolated single colonies. Suspected colonies were further

checked through Gram staining and a series of biochemical tests, including sugar fermentation, catalase activity, indole production, and the Voges–Proskauer test[2,51]. Thereafter, pure *E. coli* colonies of 116 presumptive isolates (wild fish = 64 and cultured fish = 52) were preserved in 20% glycerol and stored at – 20 °C for future use.

Molecular detection of *E. Coli*

The isolates were molecularly confirmed as *E. coli* using a species-specific PCR (**Table S5**) method targeting the *malB* gene, which encodes the maltose-binding protein. This gene is specific to *E. coli*and serves as a reliable marker for its identification[52]. PCR amplification was carried out using *malB*F (5′-GACCTCGGTTTAGTTCAC AGA-3′) and *malB*R (5′- CACACGCTGACGCTGACCA-3′) primers[2,50], and the presence of a PCR product of the expected size confirmed the identity of the isolates as *E. coli*. Genomic DNA was extracted from an overnight culture using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The quality and quantity of the extracted DNA were assessed using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). In each PCR around 50 ng DNA was used as template, while the primer concentration was 20 pmol for each. DNA samples with A260/280 and A260/230 ratios of approximately 1.80 and 2.00 to 2.20, respectively, were considered high-purity[53]and stored at – 20 °C prior to PCR amplification[50,51]. The PCR amplification of the targeted DNA was conducted in a 20 µL reaction mixture in a gradient thermal cycler (Blue-Ray Biotech Corp., Taiwan). This mixture consisted of 3 µL of nuclease-free water, 10 µL of 2X master mix (Promega, Madison, WI, USA), 1 µL each of forward and reverse primers, and 5 µL of the DNA template. To ensure the accuracy of the PCR results, positive controls consisting of *E. coli*genomic DNA, which had been previously confirmed for the target genes, were included in the reaction[50]. PCR-negative controls used non-template controls, where PBS was included instead of genomic DNA. The amplified PCR products were subjected to electrophoresis on a 1.5% agarose gel and visualized using an ultraviolet transilluminator (Biometra, Göttingen, Germany). A 100 bp DNA ladder (Promega, Madison, WI, USA) was utilized to verify the expected sizes of the PCR products[49,50]. Finally, 87 isolates, comprising 45 from wild fish and 42 from cultured fish, were identified as *E. coli* through species-specific PCR.

Biofilm formation in *E. Coli*

Qualitative method

The qualitative assessment of biofilm-producing *E. coli* was conducted using the CRA plate method. In this method, *E. coli* isolates ($N = 87$) were cultured on CRA plates, which contain Congo Red dye. The biofilm production capability of the isolates was evaluated based on the phenotypic characteristics of the colonies (**Fig. S2**)[54]. To prepare CRA plates, 56 g of Brain Heart Infusion (BHI) agar was dissolved in 1 L of double-distilled water. After adding 0.8 g of Congo Red dye and 36 g of sucrose, the solution was gently heated until all components were fully dissolved. The mixture was then sterilized by autoclaving at 121 °C and 15 per square inch (psi) for 30 min[51]. Once autoclaved, the media was poured into sterilized Petri dishes, allowed to cool to room temperature, and incubated to ensure sterility. For assessing biofilm formation, a loopful of bacterial suspension was streaked onto the CRA plates and incubated overnight at 37 °C, followed by an additional 24 h at room temperature. Colonies that appeared as robust, crusty, black were classified as strong biofilm producers, while red colonies were identified as non-biofilm-producing strains. All the experiments were repeated twice.

Quantitative method

For the quantitative assessment of biofilm production by *E. coli* ($N = 87$ isolates), the CVMT method, as described by Rana et al.[48], was employed. A single colony from a CRA plate was inoculated into a 1.5 mL Eppendorf tube containing nutrient broth supplemented with 2% sugar and incubated overnight at 37 °C. Subsequently, the biofilm formation assay was performed using a 96-well microtiter plate. Each well was filled with 180 µL of Tryptic Soy Broth (TSB) supplemented with 2% sugar, and 20 µL of the adjusted overnight culture (McFarland 0.5 standard) was added[51]. The microtiter plate was incubated at 37 °C for 24 h. A control well containing 200 µL of TSB without test samples was included for comparison. After incubation, the wells were thoroughly washed with distilled water to remove non-adherent planktonic bacteria. The biofilms were then stained with 1% crystal violet, and the plates were allowed to air dry. Biofilm quantification was achieved by measuring the absorbance at 570 nm using an ELISA reader[24]. The data were interpreted according to established criteria for biofilm formation.

Antibiotic susceptibility assay

The Antibiotic Sensitivity Test (AST) of the PCR-positive *E. coli* isolates ($N = 87$) were assessed using the Kirby-Bauer disk diffusion method (DDM)[55], in accordance with the guidelines outlined in the Clinical Laboratory Standards Institute (CLSI) M100 33rd Edition[56]. We selected the ten most frequently used antibiotics in Bangladesh from different groups.

These were ciprofloxacin (CIP, 5 µg), gentamicin (GEN, 10 µg), tetracycline (TET, 30 µg), ceftriaxone (CTR, 30 µg), ampicillin (AMP, 25 µg), ceftazidime (CAZ, 5 µg), chloramphenicol (C, 30 µg), imipenem (IMP, 10 µg), fosfomycin (FOS, 50 µg), and nitrofurantoin (NIT, 300 µg). The isolated colonies were taken into 4–5 mL of nutrient broth for performing DDM. After preparing the broth cultures, they were incubated for 4–5 h at 37 °C, and the turbidity of bacterial suspensions was adjusted with the 0.5 McFarland unit (HiMedia, India). After that, the dried surface of a Muller Hilton (MH) agar plate was inoculated by spreading the broth suspension on the surface with sterile cotton swabs. Finally, the antibiotic disks were applied on the surface of the agar plates and left for overnight incubation at 37 °C. The isolates were categorized as susceptible, intermediate, and resistant according to CLSI guidelines[56]. MDR patterns, defined as resistance to >3 antibiotics, were identified using the protocol i.e., any isolate not susceptible to at least one agent in at least three antimicrobial classes outlined by Sweeney et al.[57]. The Multiple Antibiotic Resistance (MAR) index was calculated by the following formula:

MAR = u/v; where, u = total number of antibiotics that an isolate showed resistance and v = total number of antibiotics used in this study[58]. *E. coli* strain ATCC25922 was used as the negative control in the antimicrobial susceptibility tests.

Molecular detection of antibiotic resistance and biofilm forming genes in *E. Coli*

To identify antibiotic resistance and virulence genes in the *E. coli* isolates ($N = 87$), we performed conventional PCR assays targeting beta-lactam resistance genes (e.g., *blaTEM*, *blaSHV*, *blaCTX*, and *sul1*) and the biofilm forming gene *fimC* using specific primers (**Table S5**). Genomic DNA from *E. coli* isolates was extracted from overnight cultures using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The quality and quantity of extracted DNA were assessed as previously detailed in Sect. 2.3[50,51]. PCR was carried out using a gradient thermal cycler (Blue-Ray Biotech Corp., Taiwan). Each PCR reaction consisted of a 10 µL mixture, including 5 µL of 2X master mix (Promega, Madison, WI, USA), 2 µL of genomic DNA (50 ng/µL), 0.5 µL of each primer, and 2 µL of nuclease-free water. The PCR products were then analyzed by electrophoresis on a 1% agarose gel. After amplification, the amplicons were stained with ethidium bromide and visualized under an ultraviolet transilluminator (Biometra, Göttingen, Germany). A 100 bp DNA ladder (Promega, Madison, WI, USA) was used to verify the size of the PCR amplicons[49,50]. Although, a positive control was not used in the PCR for resistance and virulence genes, non-template control (NTC, no template DNA) was used as a negative control.

Statistical analysis

Data were entered into Microsoft Excel 2020 (Microsoft Corp., Redmond, WA, USA) and analyzed using SPSS version 25 (IBM Corp., Armonk, NY, USA), Origin 2024b, GraphPad Prism version 8.4.3 (GraphPad Software, Inc.) and R packages. The Pearson's chi-square test was conducted to compare the occurrence of *E. coli*across different sample categories (e.g., koi fish and shing fish). Prevalence percentages were calculated by dividing the number of positive samples in each category by the total number of samples tested within that category[59,60]. The prevalence formula was applied for determining occurrence percentage of *E. coli*. The AMR patterns, resistance, intermediate and sensitivity, and MAR index were calculated using the CLSI (2023) guideline using the cut-off as provided in the brochure of the manufacturer (Liofilchem, Italy).

Data availability

All data generated or analyzed during this study are included in this article [and its supplementary information files].

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Author contributions
M.L.R., M.N.H. and M.T.R. conceived and designed the study; M.L.R., and M.A.U. acquisition of data, analysis and writing original draft; M.N.H., J.H., M.P.S. and M.T.R. critical review and editing. All authors contributed to the article and approved the submitted version.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
The animal study protocol was approved by the Ethics Committee of Bangladesh Agricultural University (BAU) in Mymensingh, Bangladesh [AWEEC/BAU/2023(25)]. All experimental procedures and methods were conducted in strict compliance with relevant guidelines and regulations, and the findings were documented in accordance with the ARRIVE guidelines (https://arriveguidelines.org). Notably, this study did not involve the use of anesthesia or euthanasia methods.

Additional information
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