

Biofilm Formation, Multicellular Behavior, and Antimicrobial Susceptibility of *Escherichia coli* Strains Isolated from Fish in Ajmer, Rajasthan

Ashish Sahu

Model Rural Health Research Unit (ICMR-NIIRNCD) Bhanpur Kalan, Jaipur, Rajasthan, India-302028

Corresponding Author: Dr. Ashish Sahu

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ABSTRACT

Foodborne illnesses remain a major global public health concern, with fish often acting as a carrier of pathogenic bacteria such as *Escherichia coli* (*E. coli*). The misuse of antibiotics in aquaculture has further increased the risk of antibiotic resistance, making this issue more urgent. In the present study, fish fecal samples were collected from local markets in Ajmer, Rajasthan, to isolate and characterize *E. coli*. The isolates were identified using EMB agar and confirmed through biochemical (IMViC) tests, followed by molecular confirmation with PCR amplification of the 16s rRNA gene. Biofilm formation was observed among the isolates, with different morphotypes (RDAR, BDAR, SAW, PDAR) detected. Antimicrobial susceptibility testing revealed varied resistance patterns, with several isolates showing resistance to commonly used antibiotics such as ceftazidime and ciprofloxacin, while others remained sensitive to drugs like meropenem and colistin. The findings highlight the presence of biofilm-forming, multidrug-resistant *E. coli* strains in fish, posing a potential health risk to consumers. This study underscores the need for stricter monitoring of antibiotic use in aquaculture and better food safety practices to prevent the spread of resistant pathogens through the food chain.

Keywords: Foodborne illnesses, Biofilm Formation, Multicellular Behavior, Antimicrobial Susceptibility, *E. coli*.

INTRODUCTION

Foodborne diseases represent a persistent and growing challenge to global public health (Tauxe, et al., 2010). The World Health Organization (WHO) has consistently reported that millions of people suffer from illnesses each year due to the consumption of contaminated food and water (Alijanzadeh, et al., 2023). Bacterial pathogens are among the most common culprits, and they are often transmitted through animal-derived food products (Ali & Alsayeqh, 2022). Contaminated fish and seafood have been identified as important reservoirs of enteric bacteria, including *Escherichia coli* (*E. coli*), which can pose a direct health risk to consumers (Adedeji et al., 2012). *E. coli* is widely recognized as an indicator of fecal contamination in food and water systems (Khan, 2020). While many strains are harmless commensals, pathogenic variants can cause severe gastrointestinal infections and, in some cases, systemic disease (Maier et al., 2024). The consumption of fish carrying pathogenic *E. coli* therefore represents not only a food safety issue but also a broader public health concern (Panel on Biological Hazards, 2015). In India and other countries where aquaculture plays a vital role in food

supply, the risk of bacterial contamination is compounded by widespread antibiotic use (Okocha et al., 2018). Farmers often rely on antibiotics to prevent infections in fish stocks, enhance growth, and reduce economic losses. However, this practice has unintentionally accelerated the emergence of antibiotic-resistant bacteria in aquatic environments (Hossain et al., 2022).

Antimicrobial resistance (AMR) has been recognized as one of the most urgent health threats of the 21st century (Coque et al., 2023). Resistant bacteria originating from aquaculture can enter the human food chain through consumption of contaminated fish, handling of raw products, or contamination of water sources used in farming (Yuan et al., 2023). Once established, resistance traits can spread rapidly across bacterial populations, limiting treatment options, and leading to higher morbidity and mortality in affected individuals (Livermore, 2003). This “farm-to-fork” pathway underscores the importance of monitoring aquaculture systems for resistant pathogens, including *E. coli* (Brauge et al., 2024).

An additional factor that makes *E. coli* particularly problematic is its ability to form biofilms (Sharma et al., 2016). Biofilms are structured microbial communities encased in extracellular polymeric substances that adhere to surfaces, including fish tissue, equipment, and aquatic environments (Fish et al., 2016). In this protected state, bacteria become significantly less susceptible to environmental stressors, disinfectants, and antimicrobial agents (Rozman et al., 2021). Biofilm-associated *E. coli* not only persist longer in aquatic systems but also pose greater challenges for eradication during food processing (Rather et al., 2021). Moreover, biofilm formation is closely linked to antibiotic resistance, with biofilm-associated bacteria often requiring much higher concentrations of antimicrobials for effective inhibition (Algburi et al., 2017).

Given this background, the present study was designed to provide an in-depth assessment of *E. coli* strains isolated from

fish samples collected in Ajmer, Rajasthan. Specifically, the study sought to:

1. Isolate and characterize *E. coli* using selective culture and biochemical assays.
2. Evaluate their ability to form biofilms and identify associated morphotypes.
3. Assess antimicrobial susceptibility patterns against commonly used antibiotics.
4. Confirm the bacterial identity at the molecular level using PCR-based methods.

By combining phenotypic, biochemical, and molecular approaches, this research contributes to a better understanding of the public health risks posed by *E. coli* in fish sold in local markets. The findings are expected to inform both food safety policies and strategies to mitigate antimicrobial resistance in aquaculture.

MATERIALS & METHODS

Sample Collection

Fish fecal samples were collected from local markets near railway stations in Ajmer, Rajasthan. A total of 21 samples were obtained under aseptic conditions and transported to the laboratory in sterile containers within 2–3 hours of collection. Samples were stored at 4 °C and processed within 24 hours to minimize changes in microbial composition.

Isolation and Identification of *E. coli*

The samples were streaked on Eosin Methylene Blue (EMB) agar, a selective and differential medium for *E. coli*. Colonies showing the characteristic green metallic sheen were presumptively identified as *E. coli*. Pure isolates were further confirmed by standard biochemical tests (IMViC: Indole, Methyl Red, Voges-Proskauer, and Citrate utilization). The IMViC pattern was recorded and compared with reference strain characteristics for *E. coli*.

Biofilm Formation Assay

Biofilm formation among *E. coli* isolates was assessed based on colony morphology

on Congo Red agar plates. The isolates were categorized into different morphotypes: RDAR (red, dry, and rough), BDAR (brown, dry, and rough), SAW (smooth and white), and PDAR (pink, dry, and rough). Each isolate was incubated under identical conditions, and morphotype expression was documented.

Antimicrobial Susceptibility Testing

The antibiotic resistance profile of the isolates was determined using the standard Kirby–Bauer disk diffusion method, following CLSI (Clinical and Laboratory Standards Institute) guidelines. The antibiotics tested included:

- Meropenem (10 µg)
- Amikacin (30 µg)
- Colistin (10 µg)
- Levofloxacin (5 µg)
- Ceftazidime (30 µg)
- Ciprofloxacin (5 µg)

After incubation at 37 °C for 18–24 hours, zones of inhibition were measured and interpreted as Sensitive (S) or Resistant (R) according to CLSI breakpoints.

Molecular Characterization

For molecular confirmation, DNA was extracted from pure isolates using the standard boiling method. Polymerase Chain Reaction (PCR) was carried out targeting the 16s rRNA gene with universal primers. Amplified products were resolved on a 1.5% agarose gel stained with ethidium bromide and visualized under UV transillumination. A 100 bp DNA ladder was used as a molecular weight marker.

STATISTICAL ANALYSIS

Descriptive statistics were used to summarize biofilm morphotypes and antibiotic susceptibility profiles. The frequency and percentage of resistant isolates were calculated for each antibiotic.

RESULT

Isolation and Identification of *E. coli*

Fish fecal samples collected from local markets in Ajmer yielded multiple bacterial

colonies on EMB agar. Colonies showing the characteristic metallic green sheen were presumptively identified as *E. coli* (Fig. 1). Pure cultures were obtained and subjected to biochemical identification using IMViC tests (Fig. 2). The isolates demonstrated positive reactions for indole and methyl red tests, and negative reactions for Voges–Proskauer and citrate utilization, consistent with the reference profile of *E. coli* (Figs. 3–6).



Fig.1 Mixed colony of bacteria on EMB plate.



Fig.2 Isolated *E. coli* Pure culture on EMB plate.

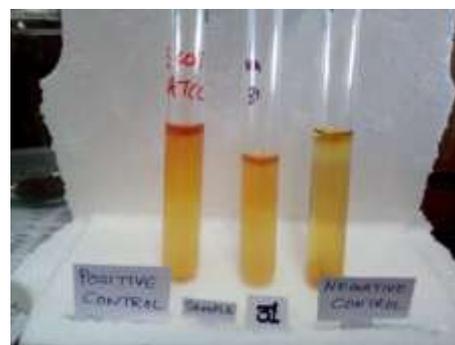


Fig. 3 Indole test result

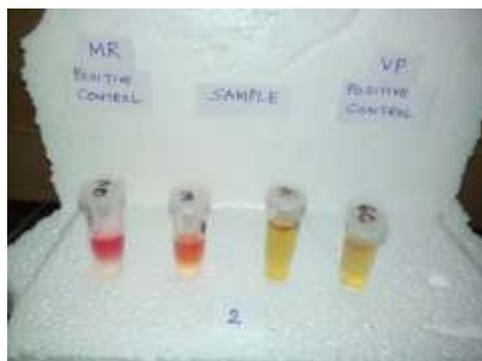


Fig.4 Methyl red test result.



Fig. 6 Citrate utilization Test result.

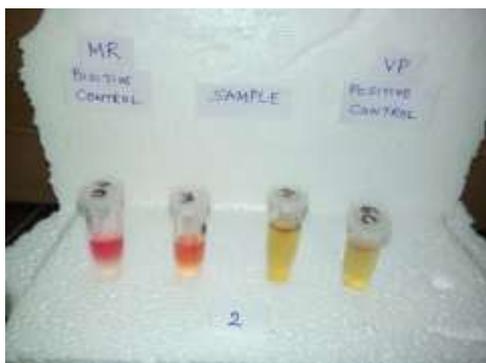


Fig. 5 Voges-Proskauer Test result

Biofilm Formation

The biofilm-forming ability of *E. coli* isolates was evaluated on Congo Red agar. A total of 21 isolates were analyzed and classified into distinct morphotypes: RDAR (red, dry, and rough), BDAR (brown, dry, and rough), SAW (smooth and white), and PDAR (pink, dry, and rough). RDAR was the most frequently observed phenotype, followed by BDAR, whereas SAW and PDAR were less common.

Biofilm Formation

<i>E. coli</i> isolates	Morphology	<i>E. coli</i> isolates	Morphology
1	SAW	22	BDAR
2	SAW	23	RDAR
3		24	BDAR
4		25	PDAR
5	BDAR	26	BDAR
6	BDAR	27	SAW
7	RDAR	28	BDAR
8	RDAR	29	RDAR
9	RDAR	30	RDAR
10	BDAR	31	RDAR
11	RDAR	32	RDAR
12	BDAR	33	SAW
13	RDAR	34	BDAR
14	BDAR	35	RDAR
15	RDAR	36	PDAR
16	RDAR	37	BDAR
17	RDAR	38	BDAR
18	RDAR	39	BDAR
19	RDAR	40	RDAR
20	RDAR	41	RDAR
21	RDAR	42	SAW

Antimicrobial Susceptibility

Antimicrobial susceptibility testing of the isolates revealed variable resistance patterns. High levels of resistance were observed against ceftazidime and

ciprofloxacin, while most isolates remained sensitive to meropenem, amikacin, and colistin. Intermediate responses were seen with levofloxacin in some isolates.

Meropenem	Amikacin	Colistin	Levofloxacin	Ceftazidime	Ciprofloxacin
S 20	S 16	S 18	S 22	R	S23
S 23	S 18	S16	R	S 15	S24
S 25	R	S 19	R	R	R
R	S16	S15	R	R	R
S22	S18	S 20	R	R	R
S 19	R	S20	S16	R	S22
S21	S18	S16	S 23	R	S 25
S18	S16	S18	S18	S 16	S24
S19	S17	S17	R	R	R
R	S16	S18	R	R	R
S20	S19	S19	R	R	R
S21	S19	S20	S21	R	S23
S22	S15	S20	S21	S14	R
S19	S17	S21	R	R	R
S21	S16	S16	S19	R	S23
S23	S18	S19	R	S15	S25
S19	R	S21	R	R	R
S22	S19	S18	R	R	R
R	S16	S15	R	R	R
S18	S17	S18	R	R	R
S21	S17	S16	S22	R	S22
S24	S19	S17	S23	S16	R
S22	S18	S19	S19	S16	S24
S23	S16	S19	S17	R	S21
S19	R	S18	S21	R	S25
S25	S18	S21	S22	R	S23
S21	S15	S20	R	R	R
S22	S19	S18	R	R	R
S19	S17	S21	S18	S14	R
S20	R	S19	R	S16	R
S22	R	S17	S22	R	S24
S21	S18	S18	S20	R	S21
S23	S17	S17	S19	S15	R

PCR Confirmation

Molecular identification of selected isolates was carried out by PCR amplification of the 16s rRNA gene. The expected product size (~1500 bp size) was successfully amplified

in all tested isolates, confirming their identity as *E. coli*. Gel electrophoresis images showed clear bands for each isolate compared to the reference strain (ATCC).

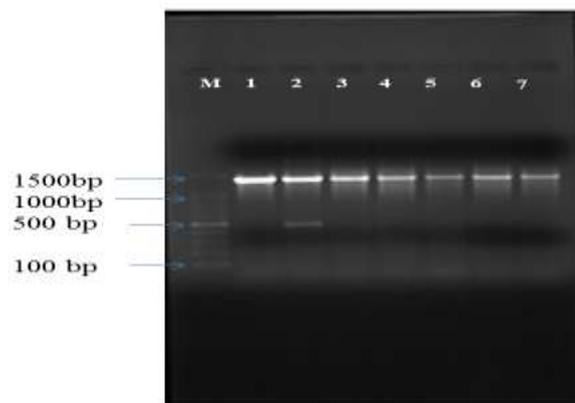


Fig.7: PCR of 16s gene of reference and samples, Lane M-100bp size ladder, lane1-ATCC, lane2- ECP1, lane3-ECP2, lane4-ECP3, lane5-ECP4, lane6-ECP5, lane7-ECP6

DISCUSSION

The present study highlights the occurrence of *Escherichia coli* in fish samples collected from local markets in Ajmer and Jaipur and provides important insights into their biofilm-forming ability, antibiotic resistance patterns, and molecular confirmation. Together, these findings shed light on the public health risks associated with the consumption of fish carrying pathogenic and resistant *E. coli*.

Prevalence of biofilm-forming *E. coli*

A notable proportion of the isolates demonstrated biofilm-forming ability, with RDAR and BDAR morphotypes being most common (Nesse et al., 2020). The predominance of these morphotypes suggests that *E. coli* in fish possess strong biofilm-forming potential, which may allow them to persist in the aquatic environment, on fish surfaces, and even within the food supply chain (Rana et al., 2025). Since biofilms confer a survival advantage by protecting bacteria from environmental stressors and antimicrobials, their presence in foodborne *E. coli* represents a major challenge for food safety and infection control (Bai et al., 2021).

Antibiotic resistance trends

The antimicrobial susceptibility testing revealed worrying resistance trends (Salam et al., 2023). Resistance to commonly used antibiotics such as ceftazidime and ciprofloxacin was high, while sensitivity was largely retained against meropenem, colistin, and amikacin (Livermore et al., 2023). These results are consistent with global reports of rising resistance to fluoroquinolones and third generation cephalosporins in foodborne *E. coli* (White, 2021). The presence of multidrug-resistant isolates in fish sold for human consumption poses a serious public health threat, as infections caused by such strains are more difficult to treat and may require the use of last-resort antibiotics (Mumbo, 2021).

Comparison with previous studies

Similar patterns of biofilm formation and antimicrobial resistance have been reported in India and internationally (Bhardwaj et al., 2021). Studies from Cochin, Chennai, and other parts of India have shown the presence of pathogenic *E. coli* in retail fish with varying degrees of resistance to commonly used antibiotics (Shafeek, 2024). Globally, research from Africa, Europe, and Southeast Asia also supports the finding that aquaculture environments act as reservoirs of resistant *E. coli*, with biofilm formation enhancing their persistence (Rana et al., 2024). This consistency across studies highlights the widespread nature of the problem and the urgent need for global surveillance.

Public health concerns

The findings carry several implications for public health. First, the consumption of fish contaminated with resistant *E. coli* may serve as a direct transmission route of antimicrobial resistance to humans. Second, biofilm formation increases the survival of these bacteria during food processing, storage, and cooking, raising the likelihood of infection. Third, resistant *E. coli* in aquaculture environments may transfer resistance genes horizontally to other bacterial species, further compounding the AMR crisis. The risk is particularly high in communities where fish is a staple protein source and antibiotic use in aquaculture is poorly regulated (Lulijwa et al., 2024).

Possible sources of resistance

The resistance observed in this study is likely linked to multiple factors, including the prophylactic use of antibiotics in aquaculture to prevent disease outbreaks, contamination of aquatic environments through sewage or agricultural runoff, and the misuse of antibiotics in veterinary and human medicine (Mo et al., 2017). Together, these practices create selective pressure that fosters the emergence and persistence of resistant strains in fish populations (Derome et al., 2017).

Limitations and future directions

This study is not without limitations. The sample size was relatively modest and restricted to two geographic locations, which may limit the generalizability of findings. Additionally, while phenotypic resistance was assessed, the specific resistance genes were not identified. Future research should focus on larger-scale epidemiological surveys across multiple regions and incorporate molecular techniques such as sequencing to detect resistance determinants. Such studies would provide a more comprehensive understanding of the genetic basis of resistance and its potential for horizontal transfer.

Hence, the study demonstrates that fish sold in Ajmer and Jaipur markets harbor biofilm-forming, multidrug-resistant *E. coli*. These findings underscore the urgent need for stricter regulation of antibiotic use in aquaculture, improved hygiene in fish markets, and routine monitoring of foodborne pathogens to safeguard consumer health.

CONCLUSION

This study demonstrated that fish sold in local markets in Ajmer and Jaipur harbor *Escherichia coli* strains with strong biofilm-forming ability and concerning levels of antimicrobial resistance. RDAR and BDAR morphotypes were the most prevalent, indicating a high potential for persistence in aquatic and food environments. Antimicrobial susceptibility testing revealed multidrug resistance, particularly against commonly used antibiotics such as ceftazidime and ciprofloxacin, while sensitivity to meropenem, colistin, and amikacin was largely retained. Molecular confirmation through PCR amplification of the 16s rRNA gene further validated the identity of the isolates.

The presence of biofilm-forming, multidrug-resistant *E. coli* in fish highlights a significant public health risk, as these pathogens can be transmitted through the food chain and may compromise treatment

options in clinical settings. The findings underscore the urgent need for systematic monitoring of bacterial contamination in aquaculture and retail fish markets. Stricter regulation of antibiotic use in fish farming, along with improved hygiene and food safety practices, are essential to reduce the spread of resistant pathogens and safeguard consumer health.

Declaration by Author

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