emergent
Life Sciences Research

Research Article

Comparative analysis of oxytetracycline and chloramphenicol on mutation frequency in *Escherichia coli* isolates from aquatic environments

Qurratul Ain Qureshi, Asif Majeed Mir, Asad Khan

Received: 16 November 2024
Accepted: 20 february 2025
Online: 03 March 2025

Authors:
Q. A. Qureshi
Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata, West Bengal, India

A. M. Mir
Department of Veterinary Pharmacology and Toxicology, College of Veterinary Sciences and Animal Husbandry, Rewa, Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

A. Khan
Division of Animal Genetics and Breeding, ICAR-National Dairy Research Institute, Karnal, India

asadjouravi@gmail.com

Emer Life Sci Res (2025) 11(1): 8-20

E-ISSN: 2395-6658
P-ISSN: 2395-664X

DOI: https://doi.org/10.31783/ELSR.2025.1110820

Abstract

Aquaculture's rapid expansion raises significant concerns regarding antibiotic resistance, particularly in freshwater systems where *Escherichia coli* (*E. coli*) serve as a key indicator of fecal contamination. This study investigates mutation frequencies of *E. coli* strains from aquatic environments under selective pressure from oxytetracycline and chloramphenicol. Given that *E. coli's* antibiotic resistance is a growing public health issue, understanding mutation dynamics is crucial. We evaluated mutation frequencies in five *E. coli* strains isolated from fish, water, and sediment samples. These strains were initially sensitive to chloramphenicol and oxytetracycline. The mutation rates for oxytetracycline ranged between 4.12 $x10^{-8}$ and 5.52 x 10^{-8}, while chloramphenicol exposure led to mutation frequencies from 1.19 x 10^{-4} to 9.62 x 10^{-5}. Cluster analysis using hierarchical methods revealed distinct patterns: *E. coli* strains exposed to oxytetracycline exhibited more uniform mutation frequencies compared to those exposed to chloramphenicol. The proximity matrix and dendrogram analysis demonstrated that oxytetracycline-treated strains clustered closely together, indicating similar mutational responses. Conversely, chloramphenicol-treated strains displayed greater variability, suggesting that chloramphenicol causes more diverse genetic changes in *E. coli*. The findings highlight an alarming increase in mutation rates, especially under chloramphenicol exposure, which significantly exceeds typical environmental bacterial mutation rates. These findings indicate a significant risk of antibiotic resistance and underscore the need for strict regulations and regular monitoring in aquaculture to manage it. Future research should investigate the specific mutations, strain genomics, and environmental factors contributing to resistance to address public health concerns related to antibiotic-resistant bacteria.

Keywords antibiotic resistance, aquaculture, bacteria, cluster analysis, mutation

Introduction

Aquaculture is the fastest-growing animal production sector, contributing significantly to global fish production and providing essential proteins for human consumption [1-2]. Over 80% of aquaculture is from small-scale producers, mainly in developing countries, predominantly in freshwater systems [3]. Indian aquaculture, primarily freshwater, contributes over 95% of the global annual production, with China leading in total harvested fish [2]. West Bengal ranks second in India's fishery production, with significant contributions to inland production [4-5]. Quality assurance in fish consumption addresses risks like disease agents, contaminants, and pollutants

[6]. *Escherichia coli* (*E. coli*) indicates fecal contamination in water, affecting fish and shrimp safety [7]. While most strains are harmless, virulent types can cause severe illness [8-9]. Fish with fecal contamination pose greater health risks, potentially harboring human-specific pathogens [10]. *E. coli* thrives in moist areas like soil and water [11-12]. Human pathogens, including *Salmonella* spp., *E. coli*, and others, have been linked to fish [13]. Fish in polluted water may carry bacteria, leading to common infections [14-15]. Certain *E. coli* strains pose emerging zoonotic threats [16]. Though not native to fish microbiota, *E. coli* can be present due to contaminated environments, posing public health risks in fish farming [17]. Aquaculture's rapid growth prompts concerns over excessive antibiotic use, fostering resistance in fish and gene transfer risks to humans [18]. Global antibiotic usage in food-animal production surpasses human consumption [19], with regulations differing widely [20-21]. Antibiotic resistance undermines aquaculture efficacy, with potential transmission to humans and terrestrial animals [22]. India, a major veterinary drug producer, lacks proper antibiotic regulation [23], fostering bacterial resistance and gene transfer [24]. Antibiotic-resistant *E. coli* in fish is a global concern [25], driven by irrational animal antibiotic use [26] and human treatments, leading to multidrug-resistant bacteria contamination [27]. Europe reports varying chloramphenicol resistance in fish *E. coli* [28], while fluoroquinolone use correlates with ciprofloxacin resistance [29]. *Escherichia coli* serve as reservoirs for resistance genes, reflecting antibiotic selection pressure and predicting resistance issues in pathogens [30]. Transmission of resistance, especially through food, is significant, with emerging resistant pathogens like extended beta-lactamase and transmissible quinolone-resistant *E. coli* posing infection risks [19, 31-32]. The emergence of hybrid plasmids raises additional concerns regarding both resistance and virulence. Multi-drug resistant (MDR) *E. coli* is the most significant in the food animal industry, acting as reservoirs for resistance spread through contaminated food to humans [33]. Given the evolving nature of resistance, monitoring *E. coli's* antimicrobial attributes in farmed fish and their environment is essential. While previous studies have examined antibiotic resistance in aquaculture, comparative mutation dynamics between chloramphenicol and oxytetracycline have not been thoroughly evaluated. Thus, this study aims to determine mutation frequency of *E. coli* against oxytetracycline (OTC) and chloramphenicol.

Methodology

Bacterial strains

A total of 5 chloramphenicol and oxytetracycline-sensitive *E. coli* strains from the collections of the Department of Aquatic Animal Health, Faculty of Fishery Sciences, Kolkata, were subjected for mutation frequency estimation. These strains were already profiled for their resistance against twelve potentially valuable antibiotics, *viz*., amoxyclav (A), azithromycin (Az), cephalexin (Cn), chloramphenicol (C), ciprofloxacin (Ci), co-trimoxazole (Co), enrofloxacin (E), gentamycin (G), nitrofurantoin (Ni), oxytetracycline (O), sulfafurazole (S) and trimethoprim (T) were determined from the antibiogram data. Multiple antibiotic resistance (MAR) or multiple drug resistance (MDR) is defined as resistant to three or more classes of antimicrobial [18, 34]. The Details about the source and antibiotype of each bacterial strain has been given in Table 1.

Estimation of mutants sensitive to oxytetracycline and chloramphenicol

Five chloramphenicol and oxytetracycline sensitive strains of *E. coli* isolated from fish, water and sediment samples were used for determining the frequency of mutation against the antibiotics, *viz*., chloramphenicol and oxytetracycline. Young cultures of these bacterial strains grown in 300 ml nutrient broth (NB) were were harvested and washed twice with sterile saline (0.85 % (w/v) NaCl) by centrifugation at 6000 rpm for 10 min each. The deposits were then re-suspended in 5 ml sterile saline to a concentration of about 10^{10} cells/ml. Cell suspensions were freshly prepared as and when required and used immediately. Before use, the cell suspensions were vigorously shaken in a vortex mixer to break the bacterial aggregates. Stock solutions of oxytetracycline and chloramphenicol, each at a concentration of 1000 µg/ml, were freshly prepared. Oxytetracycline dihydrate (0.02 g) was aseptically dissolved in 20 ml of sterile distilled water. Chloramphenicol powder (0.02 g) was aseptically dissolved in a 1:10 ethanol solution, consisting of 2 ml of

Table 1. Details of source, sample and antibiotype of bacterial strain

Bacterial strain	Source	Sample	Antibiotype
E. coli JCC2	Buderhat bhery	*Catla catla*	A^S,Az^I,Cn^R,C^S,Ci^R,Co^S,E^S,G^I,N^I,O^S,S^S,T^S
E. coli JGC2	Garia market	*Catla catla*	A^I,Az^R,Cn^R,C^S,Ci^R,Co^S,E^R,G^R,N^S,O^S,S^S,T^S
E. coli QBFR2	Barrackpore market	*Labeo rohita*	A^R,Az^R,Cn^R,C^S,Ci^S,Co^S,E^R,G^R,N^S,O^S,S^S,T^S
E. coli BFM2	Barrackpore market	*Cirrhinus mrigala*	A^S,Az^R,Cn^R,C^R,Ci^S,Co^S,E^S,G^R,N^S,O^S,S^S,T^S
E. coli BFW1A	Barrackpore farm	Water	A^S,Az^R,Cn^R,C^S,Ci^R,Co^R,E^S,G^R,N^I,O^S,S^S,T^R

A: Amoxyclav (30 µg), Az: Azithromycin (15 µg), Cn: Cefalexin (30 µg), C: Chloramphenicol (30 µg), Ci: Ciprofloxacin (5 µg), Co: Co- trimoxazole (25 µg), E: Enrofloxacin (10 µg), G: Gentamycin (10 µg), Ni: Nitrofurantoin (300 µg), O: Oxytetracycline (30 µg), S: Sulfafurazole (300 µg), T: Trimethoprim (10 µg); R: Resistant; I: Intermediate; S: Sensitive

absolute ethanol and 18 ml of sterile distilled water. Both stock solutions were stored in a cool, dark place until use. A concentration of 25 µg/ml for OTC and chloramphenicol was chosen to reflect realistic levels of antibiotics found in aquatic environments contaminated by agricultural runoff, aquaculture or pharmaceutical effluents. This concentration effectively simulates environmentally relevant conditions while providing sufficient selective pressure to study mutation frequency in *E. coli*. It is high enough to challenge bacterial populations without inhibiting growth and aligns with concentrations used in previous studies, ensuring comparability and reproducibility [24, 35-37]. Plates of desired concentration of both chloramphenicol and OTC (25 µg/ml each) were prepared by mixing calculated volumes of the antibiotic stock solutions with tempered Mueller-Hinton agar (MHA) before pouring. The prepared plates of desired antibiotic concentrations were stored in a refrigerator for at least 3 days. Prior to use, the plates were removed from the refrigerator and thawed to room temperature.

Mutation frequency was estimated by the method of Barnes et al., [38] with little modifications. In brief, freshly prepared bacterial cell suspension was serially diluted up to 10^{-8} level in saline. Aliquots (0.1 ml) of cell suspension from undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions were then spread on to the MHA plates containing chloramphenicol and/or OTC at 25 µg/ml. Simultaneously about 0.1 ml each of 10^{-7} and 10^{-8} dilutions of cell suspension was spread plated on to the antibiotic-free MHA plates. The seeded agar plates were incubated at 35 ± 2°C for 5-7 days and the number of growing colonies on the plates was counted. Mutation frequency was calculated as given below:

Mutation frequency of chloramphenicol sensitive *E. coli* = Nac /Nc, where,
Na^c = Number of colonies on MHA plates containing chloramphenicol
N^c = Number of colonies on antibiotic-free MHA plates
and,
Mutation frequency of oxytetracycline sensitive *E. coli* = Na^o /N^o, where,
Na^o = Number of colonies on MHA plates containing oxytetracycline
N^o = Number of colonies on antibiotic-free MHA plates

Cluster analysis

The mutation frequency values for each *E. coli* strain under oxytetracycline and chloramphenicol exposure were analyzed using hierarchical cluster analysis. The optimal number of clusters (k) was determined using the elbow method in DATAtab software. Hierarchical clustering was performed in IBM SPSS software version 25.0, employing the between-group linkage method with squared. Euclidean distance as the interval measure. The analysis included the estimation of the proximity matrix, agglomeration schedule, and case-wise determination of clusters based on average linkage between groups. Cluster membership was assigned by inputting the optimal number of clusters from the elbow method into the "single solution" option in the "Save" tab, with clustering based on the average linkage method. A dendrogram illustrating the clustering structure was also generated. To validate the classification, cluster means were compared using one-way analysis of variance (ANOVA), and silhouette coefficients were calculated to assess the robustness of the clustering results.

Results and Discussion

The frequency of mutation among the *E. coli* strains against oxytetracycline and chloramphenicol are shown in Table 2. The mutation frequency to OTC (25 µg/ml) was in the range of 4.12 x 10^{-8} in *E. coli* BFW1A - 5.52 x 10^{-8} in *E. coli* QBFR2. The mutation frequency to chloramphenicol (25 µg/ml) was in the range of 1.19 x 10^{-4} in *E. coli* JGC2 - 9.62 x 10^{-5} in *E. coli* JCC2.

Table 2. Frequency of mutation among *Escherichia coli* strains against oxytetracycline (OTC) and chloramphenicol (C)

Bacterial strain	Antibiotic tested	Mutation frequency
E. coli JCC2	OTC	5.12 x 10^{-8}
	C	9.62 x 10^{-5}
E. coli JGC2	OTC	4.05 x 10^{-8}
	C	1.19 x 10^{-4}
E. coli QBFR2	OTC	5.52 x 10^{-8}
	C	3.75 x 10^{-4}
E. coli BFM2	OTC	4.94 x 10^{-8}
	C	3.61 x 10^{-4}
E. coli BFW1A	OTC	4.12 x 10^{-8}
	C	3.28 x 10^{-5}

The cluster analysis was performed on a complete dataset of five *E. coli* strains tested under two different antibiotics (OTC: Oxytetracycline treatment, C: Chloramphenicol treatment) with no missing data, using the average linkage method. The optimal number of clusters for the dataset of *E. coli* strains was determined using the elbow method. An elbow graph, generated using DATAtab software, plotted the sum of squared distance values (y-axis) against the number of clusters (k) on the x-axis (Figure 1). The "elbow" point of the graph, corresponding to k = 3, was identified as the optimal number of clusters for the analysis. Thus, during hierarchical cluster analysis in SPSS, the number of clusters was set to three while assigning cluster membership in the "single solution" option of the "Save" tab.

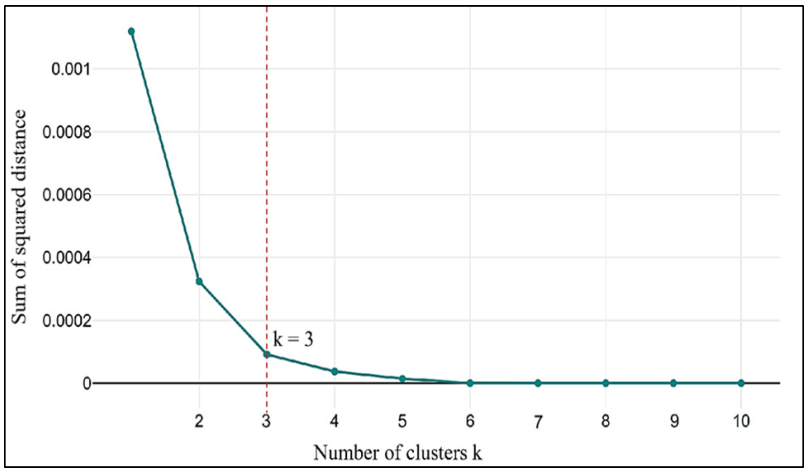

Figure 1. Elbow graph depicting the sum of squared distance values (y-axis) against the number of clusters (k) on the x-axis. The optimal number of clusters (k) is indicated at the point where the graph forms an "elbow," corresponding to k = 3

The proximity matrix in Table 3 reveals important insights into the relationships between the strains. Each value represents the squared Euclidean distance between pairs of *E. coli* strains. Cases with a squared Euclidean distance of 0.000 are identical or highly similar; for instance, Case 1 (*E. coli* JCC2 (OTC)) is identical to Cases 3, 5, 7, and 9, which are also OTC-treated *E. coli* strains, and case 3 (*E. coli* JGC2 (OTC)) is likewise identical to these cases. Larger distances indicate greater dissimilarity, such as Case 6 (*E. coli* QBFR2 (C)), which shows high dissimilarity (distance values of 14.059) from several OTC-

treated strains. Intermediate distances suggest partial similarity, with Case 2 (*E. coli* JCC2 (C)) showing moderate similarity to other OTC-treated strains (distance of 0.924). The OTC-treated strains (Cases 1, 3, 5, 7, and 9) are mostly identical or nearly so, implying that the OTC treatment has a consistent effect on these strains. In contrast, chloramphenicol-treated strains (Cases 2, 4, 6, 8, and 10) show varying degrees of similarity among themselves but are generally dissimilar to the OTC-treated strains. Case 6 is particularly distinct, highlighting a significant difference in its response to chloramphenicol compared to OTC treatment.

Table 3. Proximity matrix of squared Euclidean distances among *E. coli* strains treated with oxytetracycline (OTC) and chloramphenicol (C)

Case	Squared Euclidean Distance									
	1: *E. coli* JCC2 (OTC)	**2: *E. coli* JCC2 (C)**	**3: *E. coli* JGC2 (OTC)**	**4: *E. coli* JGC2 (C)**	**5: *E. coli* QBFR2 (OTC)**	**6: *E. coli* QBFR2 (C)**	**7: *E. coli* BFM2 (OTC)**	**8: *E. coli* BFM2 (C)**	**9: *E. coli* BFW1A (OTC)**	**10: *E. coli* BFW1A (C)**
1: *E. coli* JCC2 (OTC)	**0.000**	0.924	0.000	1.415	0.000	14.059	0.000	13.028	0.000	0.107
2: *E. coli* JCC2 (C)	0.924	**0.000**	0.925	0.052	0.924	7.773	0.924	7.012	0.925	0.402
3: *E. coli* JGC2 (OTC)	0.000	0.925	**0.000**	1.415	0.000	14.059	0.000	13.029	0.000	0.107
4: *E. coli* JGC2 (C)	1.415	0.052	1.415	**0.000**	1.415	6.554	1.415	5.856	1.415	0.743
5: *E. coli* QBFR2 (OTC)	0.000	0.924	0.000	1.415	**0.000**	14.058	0.000	13.028	0.000	0.107
6: *E. coli* QBFR2 (C)	14.059	7.773	14.059	6.554	14.058	**0.000**	14.059	0.020	14.059	11.710
7: *E. coli* BFM2 (OTC)	0.000	0.924	0.000	1.415	0.000	14.059	**0.000**	13.029	0.000	0.107
8: *E. coli* BFM2 (C)	13.028	7.012	13.029	5.856	13.028	0.020	13.029	**0.000**	13.029	10.772
9: *E. coli* BFW1A (OTC)	0.000	0.925	0.000	1.415	0.000	14.059	0.000	13.029	**0.000**	0.107
10: *E. coli* BFW1A (C)	0.107	0.402	0.107	0.743	0.107	11.710	0.107	10.772	0.107	**0.000**

Overall, the matrix shows a clear separation between the highly similar OTC-treated *E. coli* strains and the more varied chloramphenicol-treated strains, suggesting that OTC has a strong, consistent effect on these bacteria. The Agglomeration Schedule in Table 4 from the hierarchical cluster analysis of *E. coli* strains treated with Oxytetracycline (OTC) and Chloramphenicol (C) details the steps in which clusters were combined during the analysis. The "Stage" column represents the step number in the agglomeration process, with each step involving the merging of two clusters. The "Cluster Combined" column indicates the clusters that were combined at each stage, with clusters named after the specific *E. coli* strain and treatment. The "Coefficients" column shows the distance (or similarity) at which the clusters were combined, where lower coefficients indicate higher similarity. The "Stage Cluster First Appears" column notes the stage at which each cluster first appeared, with a value of 0 indicating an original cluster, and the "Next Stage" column indicates the next stage in which the newly formed cluster will participate.

The clustering process begins by combining highly similar OTC-treated *E. coli* strains, showing minimal variation among them under OTC treatment. As the process continues, chloramphenicol treated strains start to cluster together, demonstrating moderate similarity. The final stages involve merging the large OTC cluster with Chloramphenicol-treated strains, but the higher coefficients indicate that these two groups are quite distinct. In the early stages (1-4), the clusters combined are all OTC-treated strains with coefficients of 0.000, indicating identical or extremely similar clusters, resulting in a large cluster forming early in the process. In the middle stages (5-7), Chloramphenicol-treated strains begin to cluster, with stage 7 showing the large OTC-treated cluster merging with Cluster 10 (*E. coli* BFW1A (C)) at a coefficient of 0.107, indicating the start of merging between OTC-treated and Chloramphenicol-treated strains, though with a small distance. By stage 8, Cluster 1 (OTC-treated strains) merges with Cluster 2 (*E. coli* JCC2 (C)), a Chloramphenicol-treated strain, but the coefficient jumps to 1.070, showing lower similarity between these clusters. In stage 9, the final merge occurs between Cluster 1 (a combination of both OTC and

Table 4. Agglomeration schedule from hierarchical cluster analysis of mutation frequencies of *E. coli* strains under oxytetracycline (OTC) and chloramphenicol (C) treatments

Stage	Cluster Combined		Coefficients	Stage Cluster First Appears		Next Stage
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	
1	3: *E. coli* JGC2 (OTC)	9: *E. coli* BFW1A (OTC)	0.000	0	0	4
2	1: *E. coli* JCC2 (OTC)	7: *E. coli* BFM2 (OTC)	0.000	0	0	3
3	1: *E. coli* JCC2 (OTC)	5: *E. coli* QBFR2 (OTC)	0.000	2	0	4
4	1: *E. coli* JCC2 (OTC)	3: *E. coli* JGC2 (OTC)	0.000	3	1	7
5	6: *E. coli* QBFR2 (C)	8: *E. coli* BFM2 (C)	0.020	0	0	9
6	2: *E. coli* JCC2 (C)	4: *E. coli* JGC2 (C)	0.052	0	0	8
7	1: *E. coli* JCC2 (OTC)	10: *E. coli* BFW1A (C)	0.107	4	0	8
8	1: *E. coli* JCC2 (OTC)	2: *E. coli* JCC2 (C)	1.070	7	6	9
9	1: *E. coli* JCC2 (OTC)	6: *E. coli* QBFR2 (C)	11.570	8	5	0

Chloramphenicol-treated strains) and Cluster 6 (*E. coli* QBFR2 (C)) at a much larger coefficient of 11.570, indicating significant dissimilarity between these groups. This pattern suggests that treatment type strongly influences the similarity of *E. coli* strains, with OTC-treated strains being more homogeneous compared to those treated with Chloramphenicol. The hierarchical cluster analysis provided a visual representation of the similarity in mutation frequencies among different *E. coli* strains when exposed to oxytetracycline (OTC) and chloramphenicol (C), helping to identify which strains share similar mutational responses and which ones are more distinct, thereby offering insights into potential resistance mechanisms. Icicle plot in Figure 2 illustrates the results of hierarchical cluster analysis of *E. coli* strains, highlighting their mutation frequency under exposure to oxytetracycline (OTC) and chloramphenicol (C). The X-axis represents the number of clusters formed, while the Y-axis lists the different *E. coli* strains along with their respective treatments. Each bar's length corresponds to the degree of similarity a strain shares with others in its cluster, with longer bars indicating closer relationships. The graph demonstrates that *E. coli* strains treated with Oxytetracycline (OTC) consistently exhibit a high number of clusters, with strains 1 (*E. coli* JCC2), 7 (*E. coli* BFM2), 5 (*E. coli* QBFR2), 3 (*E. coli* JGC2), and 9 (*E. coli* BFW1A) showing similar cluster numbers, indicating a strong similarity in mutation frequency under OTC treatment. This consistent clustering suggests that OTC treatment leads to similar mutation patterns across these strains. In contrast, *E. coli* strains treated with Chloramphenicol (C) display more varied clustering, with strain 10 (*E. coli* BFW1A (C)) forming a high number of clusters comparable to the OTC-treated strains, while strains 2 (*E. coli* JCC2 (C)) and 4 (*E. coli* JGC2 (C)) have slightly fewer clusters, and strains 6 (*E. coli* QBFR2 (C)) and 8 (*E. coli* BFM2 (C)) show even fewer clusters, indicating greater dissimilarity or less frequent mutations under Chloramphenicol treatment. Overall, the OTC-treated strains demonstrate higher and more consistent clustering, reflecting the strong and uniform impact of OTC, whereas the Chloramphenicol-treated strains exhibit greater variability, suggesting a more diverse response to the treatment. The dendrogram in Figure 3, created using the average linkage method, calculates the distance between clusters as the average distance between all pairs of data points within them, which typically produces more balanced clusters. The rescaled distance cluster combine, used for better visualization, prevents the dendrogram from becoming too wide or narrow. The horizontal axis in the dendrogram shows the rescaled distance at which clusters merge,

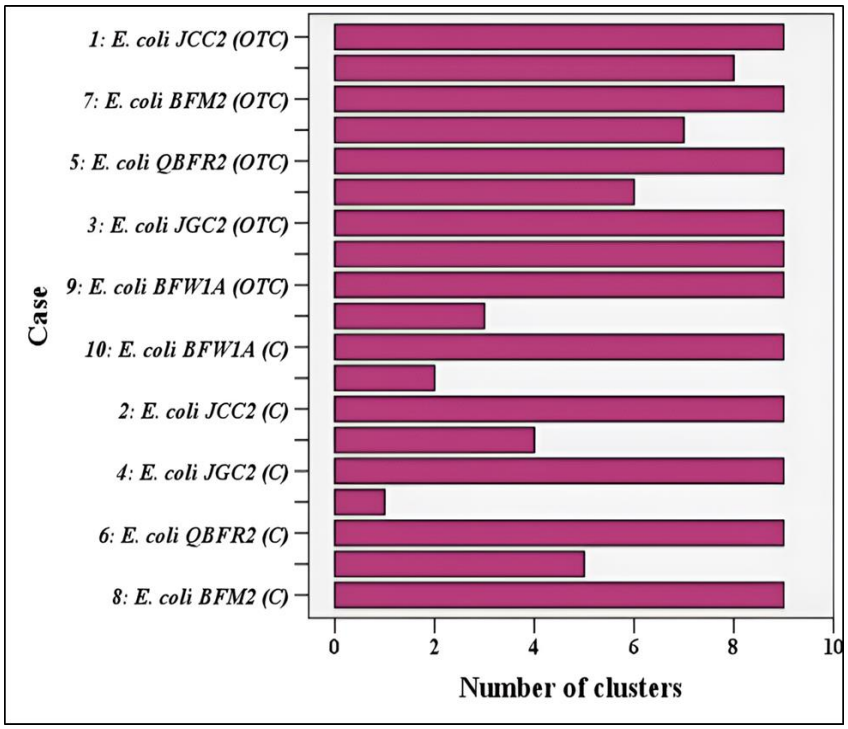

Figure 2. Icicle plot of hierarchical clustering of mutation frequencies of *Escherichia coli* strains exposed to oxytetracycline (OTC) and chloramphenicol (C)

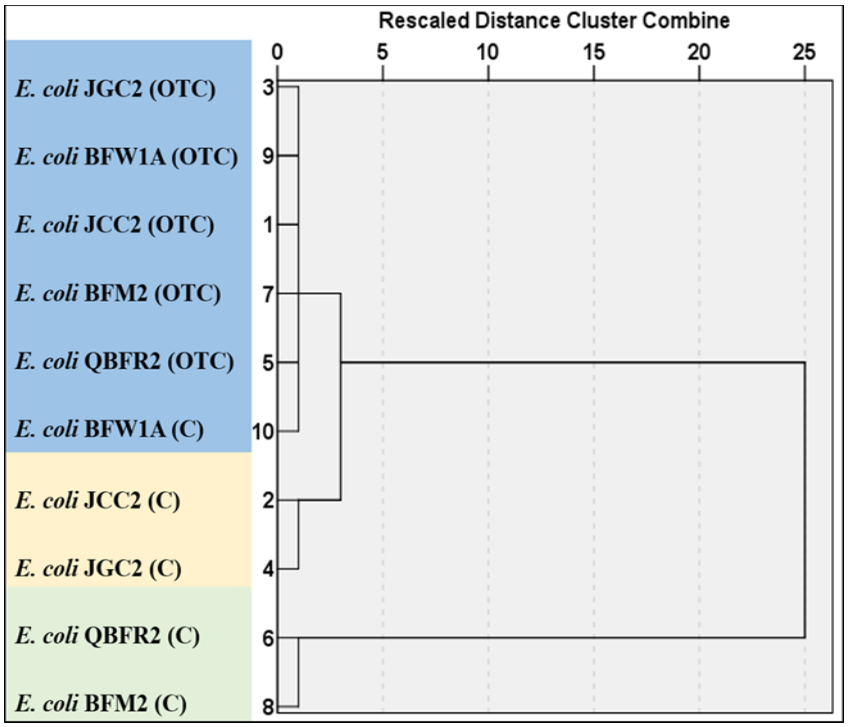

Figure 3. Dendrogram-based on average linkage between mutation frequency of *Escherichia coli* strains exposed to oxytetracycline (OTC) and chloramphenicol (C)

while the vertical lines represent the clusters formed at varying levels of similarity or distance. *E. coli* strains treated with Oxytetracycline (*E. coli* JGC2 (OTC), *E. coli* BFW1A (OTC), *E. coli* JCC2 (OTC), *E. coli* BFM2 (OTC), and *E. coli* QBFR2 (OTC)) cluster closely at a low rescaled distance, indicating a high similarity in mutation frequencies among these strains and suggesting that OTC treatment leads to similar genetic outcomes across these strains. In contrast, *E. coli* BFW1A (C), treated with Chloramphenicol, joins the OTC cluster at a moderate distance, showing some similarity to the OTC strains but not as closely as those within the OTC group. The remaining Chloramphenicol-treated strains (*E. coli* JCC2 (C), *E. coli* JGC2 (C) and *E. coli* QBFR2 (C), *E. coli* BFM2 (C), respectively) form two separate clusters at a higher rescaled distance, indicating that they are more similar to each other within their clusters than to the OTC-treated strains but still distinct from the OTC cluster.

This dendrogram reveals a clear separation between OTC and Chloramphenicol treatments, with OTC-treated strains forming a tight cluster early in the hierarchical process and Chloramphenicol-treated strains showing greater variability and clustering later at a higher distance. This structure highlights the significant role of antibiotic type in determining mutation frequency patterns, with OTC resulting in more uniform effects compared to the more variable effects of Chloramphenicol, thus providing insights into the adaptive responses of *E. coli* to different antimicrobial treatments. At the end of the hierarchical cluster analysis, with the optimal number of clusters set to three (k=3) by assigning the number of clusters as three during cluster membership, it was observed that the chloramphenicol treated strains *E. coli* QBFR2 and *E. coli* BFM2, exhibiting mutation frequencies of 3.75×10^{-4} and 3.61×10^{-4}, respectively, were grouped together in Cluster 3. Similarly, the chloramphenicol-treated strains *E. coli* JCC2 and *E. coli* JGC2, with mutation frequencies of 9.62×10^{-5} and 1.19×10^{-4}, were clustered together in Cluster 2. Meanwhile, *E. coli* BFW1A, the only chloramphenicol-treated strain, was grouped with all the oxytetracycline-treated *E. coli* strains in Cluster 1. Similar clustering pattern could also be seen in the cluster dendrogram, which showed three distinct clusters (Figure 3). The results were further validated by comparing the means of the three clusters using one-way ANOVA, and the robustness of the clustering was assessed by silhouette values. Table 5 summarizes the results of the one-way ANOVA, which revealed highly significant differences ($p < 0.001$) among all three clusters, confirming the accurate classification of *E. coli* strains into three distinct clusters. Additionally, Table 6 presents the silhouette values for each cluster, which assess the quality of the clustering in terms of cohesion and separation. The mean silhouette values were 0.897, 0.887, and 0.973 for Cluster 1, Cluster 2, and Cluster 3, respectively, indicating that, on average, the cases within all three clusters were well-separated from each other, with strong cohesion observed for most cases. The overall mean silhouette value across all clusters was 0.910, with a minimum of 0.635 and a maximum of 0.974, suggesting that the clustering

Table 5. Least square analysis of variance of mutation frequency of bacterial strains across the clusters

Variable	Sum of Squares		Mean Square		F - value	P - value
	Between clusters (2)	Within clusters (7)	Between clusters (2)	Within clusters (7)		
Mutation frequency	1.97 x 10-7	1.25 x 10-9	9.87 x 10-8	1.79 x 10-10	551.656	<0.001

Figure in the parenthesis is degree of freedom

Table 6. Silhouette values for all the clusters using Euclidean distance as the dissimilarity measure

Cluster	Silhouettes values		
	Mean	Minimum	Maximum
1 (6)	0.897	0.635	0.949
2 (2)	0.887	0.874	0.900
3 (2)	0.973	0.972	0.974
Total	0.910	0.635	0.974

Figure in parentheses represent the number of cases within each cluster

was of high quality with generally good cohesion within clusters and separation between clusters, although some variability in cohesion was noted. Overall, the silhouette values demonstrated that the clustering was robust, with Cluster 3 exhibiting the highest consistency and quality. Bacterial DNA mutations normally occur at the rate of 1 in 10^8 cells [39]. Mutation frequency of the *E. coli* strains of the present study against OTC (25 µg/ml) was in the range of 4.05 x 10^{-8} and 5.52 x 10^{-8} were for chloramphenicol (25 µg/ml), it was found to be in the range of 1.19 x 10^{-4} and 9.62 x 10^{-5}. The frequency of mutation against chloramphenicol among the tested *E. coli* strains showed alarming levels when compared to the environmental bacterial mutation rates. Contrarily, George and Levy [40] demonstrated mutation frequencies for *E. coli* strains in the range of 2.1 x 10^{-10} – 3.1 x 10^{-7} against tetracycline and 1.0 x 10^{-8} – 5.3 x 10^{-8} against chloramphenicol. In their study, the concentration of tetracycline varied from 2.0 to 5.0 µg/ml, while the concentration of chloramphenicol was fixed at 5.0 µg/ml, which might have influenced the mutation rates. The results of the present study showed *E. coli* susceptible to OTC mutating at a rate of 4.05 – 5.52 in 10^8 cells. In case of chloramphenicol, the mutation occurred at a rate of 1.19 – 96.2 in 104 cells which is quite high among the *E. coli* of the aquatic environment. *Escherichia coli* being endemic to the aquatic environment, the alarming mutation rates substantiate the high antibiotic-resistance observed in this study. As pathogens are exposed to changing stressful environments like increased antibiotic treatments, mutators are undoubtedly to increase [41]. It is plausible that resistance mechanisms arose through a series of mutations. A positive correlation between MAR and high mutation rates is expected to be frequent in natural populations of bacterial pathogens [42]. Experimental studies showed that mutators may be enriched when bacteria are exposed to a changing environment especially while repeated challenges of antibiotics are performed [43].

Cluster analysis can be performed using various statistical techniques, including connectivity-based methods like hierarchical clustering [44], centroid-based approaches such as k-means clustering [45], and density-based techniques like DBSCAN [46-48]. Despite differences in their algorithms, all these methods aim to uncover previously unknown patterns in a dataset. Each approach has its strengths and limitations, but in this research, agglomerative hierarchical clustering (AHC) was preferred, a widely used method known for its simplicity and transparency. Although there is little literature on the factors influencing the choice of clustering techniques, AHC is advantageous because it does not require prior knowledge of the optimal number of clusters, unlike k-means clustering. A key feature of AHC is the dendrogram, which visually represents clustering results and enhances interpretability. Applying AHC involves three key decisions: selecting a distance metric, choosing a linkage method, and determining the number of clusters [49]. This research employs Euclidean distance, the average linkage method, and dendrogram analysis to address these aspects. However, multiple tools exist to validate or challenge these choices, making cluster validation crucial, especially given the wide range of distance metrics, linkage methods, and techniques for determining the optimal number of clusters.

Distance metrics determine the pairwise distances between objects), with common options like Euclidean, Chebyshev (Maximum), and Manhattan (city-block) distance frequently included in clustering software [50]. This research employed squared Euclidean distance, a widely used metric known for its sensitivity to differently scaled variables but well-suited for low-dimensional datasets like the one analyzed here. Another critical aspect is the choice of a linkage method, which determines how clusters are merged. While a variety of linkage methods exist, most distance metrics can be combined with them, resulting in numerous possible clustering configurations, e.g., Ward's method, single linkage, complete linkage, and average linkage. These methods differ in how they compute distances between clusters: single linkage connects clusters based on the minimum distance between points, complete linkage uses the maximum distance, and average linkage merges clusters based on the lowest average pairwise distance [51]. This study adopted the average linkage method, as it provides a balanced approach to clustering. Since visual information is processed rapidly, dendrograms facilitate quick interpretation and pattern recognition. The number of clusters can be determined by identifying the largest fusion steps between branches and cutting the dendrogram accordingly. While this can sometimes be straightforward, in cases where fusion heights are similar, additional validation methods may be necessary [52]. In this research, the dendrogram based on the average linkage method clearly delineated the dataset into three distinct clusters (Figure 3).

Validation of clustering can be achieved using cluster validity indices, with the Silhouette index and Rand index being among the most widely used. The Silhouette index evaluates how well an object fits within its assigned cluster compared to others [53], ranging from −1 to 1, where a value of 1 indicates well-clustered data. The Rand index, on the other hand, measures the similarity between two clustering results, with values ranging from 0 to 1, where 1 signifies identical clustering [54]. In this study, the number of clusters was first validated using one-way ANOVA after determining the optimal cluster count through the elbow method, followed by an assessment with Silhouette values (Table 5 and 6). The ANOVA results indicated highly significant differences among clusters, while the Silhouette values demonstrated strong cohesion within clusters and clear separation between them, confirming the effectiveness of the clustering approach.

The findings suggest that oxytetracycline treatment results in more homogeneous groupings among *E. coli* strains, while chloramphenicol-treated strains exhibit greater variability. This variability indicates that oxytetracycline may reduce differences due to selective pressure or other antibiotic effects, leading to more consistent mutation patterns. Understanding these differences sheds light on the distinct impacts of these antibiotics on *E. coli's* genetic behavior, with oxytetracycline causing more uniform mutation patterns compared to the diverse effects of chloramphenicol. For a deeper understanding, future analysis could focus on comparing specific mutations across strains to identify common patterns or unique features linked to antibiotic resistance, examining the genetic background of the strains to see how genomic variations affect mutation patterns, and investigating the environmental conditions of the strains' isolation to assess their role in the observed mutation patterns.

Conclusion

This study highlights significant concerns regarding mutation frequency in *E. coli* strains from aquatic environments, particularly in response to oxytetracycline and chloramphenicol. The findings show alarmingly high mutation rates under chloramphenicol exposure, far exceeding typical environmental levels. These rapid mutation rates, especially with chloramphenicol, emphasize the risk of developing antibiotic resistance in *E. coli* strains within aquaculture settings, potentially worsened by widespread and unregulated antibiotic use. This poses a risk of transferring resistant bacteria to humans via the food chain. Cluster analysis shows that while some strains exhibit similar mutational responses to the antibiotics, others display distinct patterns, indicating varied genetic or environmental influences. Specifically, oxytetracycline treatment leads to more uniform mutation patterns, whereas chloramphenicol causes greater variability, suggesting that oxytetracycline might reduce differences caused by selective pressure or other antibiotic effects. Understanding these variations is essential for clarifying the distinct impacts of these antibiotics on *E. coli's* genetic behavior. Strains with similar mutation frequencies likely share resistance mechanisms, while those with different patterns may follow diverse evolutionary pathways or possess unique genetic factors. Given the importance of aquaculture in global food security and the increasing threat of antibiotic resistance, these findings underscore the need for stringent antibiotic regulations, regular monitoring of resistance patterns, and further research into the genetic drivers of these mutations.

Conflict of interest

The present research work has no conflict of interest

Acknowledgement

The authors thank the West Bengal University of Animal and Fishery Sciences, Kolkata officials for providing the necessary infrastructure to carry out the work.

References

[1] C. J. Rodgers and M. D. Furones **(2009)**. Antimicrobial agents in aquaculture: practice, needs and issues. *In*: C. Rodgers and B. Basurco (eds) The use of veterinary drugs and vaccines in Mediterranean aquaculture, vol 86. vol A. Zaragoza: CIHEAM, pp41-59. doi: 10.13140/2.1.4697.0560.

[2] F. A. O. **(2016)**. The state of world fisheries and aquaculture. Food and Agriculture Organization of the United Nations, Rome.

[3] F. A. O. **(2018)**. The state of world fisheries and aquaculture,- Meeting the Sustainable Development Goals. FAO, Rome.

[4] B. N. Paul, S. Chanda, N. Sridhar, G. S. Saha and S. S. Giri **(2016)**. Proximate, mineral and vitamin contents of Indian major carp. Indian J. Anim. Nutr., **33:** 102-107.

[5] D.O. F. **(2022)**. Department of Fisheries. Handbook of Fisheries Statistics, 2022. Ministry of Fisheries, Animal Husbandry and Dairying, Govt. of India, New Delhi.

[6] T. Hastein, B. Hjeltnes, A. Lillehaug, J. Utne Skare, M. Berntssen and A. K. Lundebye **(2006)**. Food safety hazards that occur during the production stage: challenges for fish farming and the fishing industry. Rev. Sci. Tech., **25:** 607-625.

[7] H. S. Kumar, A. Parvathi, I. Karunasagar and I. Karunasagar **(2005)**. Prevalence and antibiotic resistance of Escherichia coli in tropical seafood. World J. Microbiol. Biotechnol., **21:** 619-623.

[8] P. Feng, S. D. Weagant and M. A. Grant **(2002)**. Enumeration of *Escherichia coli* and the coliform bacteria. *In*: Merker RI (ed) Bacteriological analytical manual, 8th ed., revision A. U.S. Food and Drug Administration, College Park.

[9] I. C. M. S. F. **(2002)**. Microbiological testing in food safety management, vol 7. Kluwer Academic/Plenum Publishers, New York. (ICMSF- International Commission on Microbiological Specifications for Foods).

[10] S. A. Wawire, D. O. Miruka, N. Nelson and A. Ofulla **(2013)**. Antimicrobial susceptibility patterns of Enterobacteriaceae isolated from domesticated animals and the environment in Lake Victoria, Kenya. Ecohydrol. Hydrobiol., **13:** 246-252.

[11] S. T. Odonko and J. K. Ampofo **(2013)**. *Escherichia coli* as an indicator of bacteriological quality of water: an overview. Microbiol. Res., **4:** 5-11.

[12] S. M. Chekabab, J. Paquin-Veillette, C. M. Dozois and J. Harel **(2013)**. The ecological habitat and transmission of *Escherichia coli* O157:H7. FEMS Microbiol. Lett., **341:** 1-12.

[13] J.M. Abou-Elela and A.M. Farag **(2004)**. Bacteriological quality and metal contents of *Diplodus vuljaris* and *Siganus rivuloyus* in the eastern harbour water: A comparative study of freshly harvested and market fish. Egypt J. Aquat. Res., **30:** 216-225.

[14] P. S. Jayasinghe and R. M. A. G. G. Rajakaruna **(2005)**. Bacterial contamination of fish sold in fish market in the Central Province of Sri Lanka. J. Nat. Sci. Found. Sri Lanka, **33:** 219-221.

[15] L. Novotny, L. Dvorska, A. Lorencova, V. Beran and I. Pavlik **(2004)**. Fish: a potential source of bacterial pathogens for human beings. Vet. Med.-Czech, **49:** 343-358.

[16] K. Nagamatsu, T. J. Hannan, R. L. Guest, M. Kostakioti, M. Hadjifrangiskou, J. Binkle and K. Dodson et al., **(2015)**. Dysregulation of *Escherichia coli* α-hemolysin expression alters the course of acute and persistent urinary tract infection. Proc. Natl. Acad. Sci., **112:** E871-E880.

[17] M. V. Cardozo, C. A. Borges, L. G. Beraldo, R. P. Maluta, A. S. Pollo, M. M. Borzi and L. F. dos Santos et al., **(2018)**. Shigatoxigenic and atypical enteropathogenic *Escherichia coli* in fish for human consumption. Braz. J. Microbiol., **49:** 936-941.

[18] E. C. D. C./E. M. E. A. **(2009)**. The bacterial challenge: time to react: European Centre for Disease Prevention and Control and European Medicines Agency. chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://www.ecdc.europa.eu/sites/default/files/media/en/publications/Publications/0909_TER_The_Bacterial_Challenge_Time_to_React.pdf.

[19] F. M. Aarestrup, H. C. Wegener and P. Collignon **(2008)**. Resistance in bacteria of the food chain: epidemiology and control strategies. Expert Rev. Anti-Infect. Ther., **6:** 733-750.

[20] D. F. Maron, T. J. Smith and K. E. Nachman **(2013)**. Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. Glob. Health, 9: 48. doi: 10.1186/1744-8603-9-48.
[21] A. Pruden, D. J. Larsson, A. Amézquita, P. Collignon, K. K. Brandt, D. W. Graham and E. Topp **(2013)**. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. Environ. Health Perspect., **121:** 878-885.
[22] H. Sorum **(2006)**. Antimicrobial drug resistance in fish pathogens, *In*: F. M. Aarestrup (ed.), Antimicrobial resistance in bacteria of animal origin. ASM Press, Washington, DC., pp213-238.
[23] H. Y. Done, A. K. Venkatesan and R. U. Halden **(2015)**. Does the recent growth of aquaculture create antibiotic resistance threats different from those associated with land animal production in agriculture? AAPS J., **17:** 513-524.
[24] E. Gullberg, S. Cao, O. G. Berg, C. Ilbäck, L. Sandegren, D. Hughes, and D. I. Andersson **(2011)**. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog., **7:** e1002158. doi: 10.1371/journal.ppat.1002158.
[25] L. L. Founou, R. C. Founou and S. Y. Essack **(2016)**. Antibiotic resistance in the food chain: a developing country perspective. Front. Microbiol., **7:** 1881. doi: 10.3389/fmicb.2016.01881.
[26] M. M. Mehendiratta, M. Khan, P. Mehndiratta and M. Wasay **(2014)**. Stroke in Asia; geographical variations and temporal trends. J. Neurol. Neurosurg. Psychiatry, **85:** 1308-1312.
[27] F. Prestinaci, P. Pezzotti and A. Pantosti **(2015)**. Antimicrobial resistance: a global multifaceted phenomenon. Pathog. Glob. Health, **109:** 309-318.
[28] J. Alexander, D. Benford, A. Boobis, S. Ceccatelli, J.-P. Cravedi, A. D. Domenico, D. Doerge et al., **(2009)**. Marine biotoxins in shellfish-Summary on regulated marine biotoxins scientific opinion of the panel on contaminants in the food chain. EFSA J., **1306:** 1-23.
[29] A. M. Hammerum and O. E. Heuer **(2009)**. Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. Clin. Infect. Dis., **48:** 916-921.
[30] M. U. Rasheed, N. Thajuddin, P. Ahamed, Z. Teklemariam and K. Jamil **(2014)**. Antimicrobial drug resistance in strains of *Escherichia coli* isolated from food sources. Rev. Inst. Med. Trop. Sao Paulo, **56:** 341-346.
[31] L.-N. Xia, L. Li, C.-M. Wu, Y.-Q. Liu, X.-Q. Tao, L. Dai and Y.-H. Qi et al., **(2010)**. A survey of plasmid-mediated fluoroquinolone resistance genes from *Escherichia coli* isolates and their dissemination in Shandong, China. Foodborne Pathog. Dis., **7:** 207-215.
[32] A. R. Vieira, P. Collignon, F. M. Aarestrup, S. A. McEwen, R. S. Hendriksen, T. Hald and H. C. Wegener **(2011)**. Association between antimicrobial resistance in Escherichia coli isolates from food animals and bloodstream isolates from humans in Europe: an ecological study. Foodborne Pathog. Dis., **8:** 1. doi: 10.1089/fpd.2011.0950.
[33] A. Szmolka and B. Nagy **(2013)**. Multidrug-resistant commensal *Escherichia coli* in animals and its impact on public health. Front. Microbiol., **4:** 258. Doi: 10.3389/fmicb.2013.00258.
[34] A. P. Magiorakos, A. Srinivasan, R. B. Carey, Y. Carmeli, M. E. Falagas, C. G. Giske and S. Harbarth et al., **(2011)**. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect., **18:** 268-281.
[35] J. L. Martinez **(2008)**. Antibiotics and antibiotic resistance genes in natural environments. Science, **321:** 365-367.
[36] K. Kummerer **(2009)**. Antibiotics in the aquatic environment–a review–part I. Chemosphere, **75:** 417-434.
[37] E. Marti and J.L. Balcazar **(2013)**. Antibiotic resistance in the aquatic environment. *In*: M. Petrovic, D. Barcelo, S. Pérez (Eds.) Comprehensive analytical chemistry. Elsevier, pp671-684.
[38] A. C. Barnes, S. G. B. Amyes, T. S. Hastings and C. S. Lewin **(1991)**. Fluoroquinolones display rapid bactericidal activity and low mutation frequencies against *Aeromonas salmonicida*. J. Fish Dis., **14:** 661-667.

[39] S. Watford and S.J. Warrington **(2017)**. Bacterial DNA mutations. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing LLC. https://pubmed.ncbi.nlm.nih.gov/29083710/ (accessed on 15 January 2025).
[40] A. M. George and S. B. Levy **(1983)**. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. J. Bacteriol., **155:** 531-540.
[41] E. Denamur and I. Matic **(2006)**. Evolution of mutation rates in bacteria. Mol. Microbiol., **60:** 820-827.
[42] J. Blazquez **(2003)**. Hypermutation as a factor contributing to the acquisition of antimicrobial resistance. Clin. Infect. Dis., **37:** 1201-1209.
[43] A. Giraud, I. Matic, M. Radman, M. Fons and F. Taddei **(2002)**. Mutator bacteria as a risk factor in the treatment of infectious diseases. Antimicrob. Agents Chemother., **46:** 863-865.
[44] M. Hastie, D. D. Torrico, G. Hepworth, R. Jacob, M. Ha, R. Polkinghorne and R. D. Warner **(2022)**. Combining hierarchical clustering and preference mapping differentiates consumer preference for dry aged mutton. Meat Sci., **192:** 108890. doi: 10.1016/j.meatsci.2022.108890.
[45] J. Palczak, D. Blumenthal and J. Delarue **(2020)**. Influence of sensory complexity on preferences for novel gourmet dairy desserts. Does Berlyne's theory apply to desserts? Food Qual. Prefer., **84:** 103957.
[46] N.V. Lu, R. Tansuchat, T. Yuizono and V.N. Huynh **(2020)**. Incorporating active learning into machine learning techniques for sensory evaluation of food. Int. J. Comput. Intell. Syst., **13:** 655-662.
[47] I. Lee and J. Yang **(2009)**. Common clustering algorithms. *In*: S. D. Brown, R. Taule and B. Walczak (Eds.), Comprehensive Chemometrics, Elsevier, Oxford, England, pp577-618.
[48] A. Saxena, M. Prasad, A. Gupta, N. Bharill, O. P. Patel, A. Tiwari and M.J. Er **(2017)**. A review of clustering techniques and developments. Neurocomputing, **267:** 664-681.
[49] G. James, D. Witten, T. Hastie and R. Tibshirani **(2021)**. Unsupervised learning. In: An Introduction to Statistical Learning with Applications in R, 2nd ed., Springe.
[50] G. James, D. Witten, T. Hastie and R. Tibshirani **(2013)**. Classification. *In*: J. Gareth, D. Witten, T. Hastie and R. Tibshirani (Eds.), An Introduction to Statistical Learning with Applications in R. Springer-Verlag New York.
[51] A. Kassambara **(2017)**. Practical Guide to Cluster Analysis in R: Unsupervised Machine Learning, 1st ed. STHDA.
[52] A. Gere **(2023)**. Recommendations for validating hierarchical clustering in consumer sensory projects. Curr. Res. Food Sci., **6:** 100522. doi: 10.1016/j.crfs.2023.100522.
[53] P.J. Rousseeuw **(1987)**. Silhouettes: a graphical aid to the interpretation and validation of cluster analysis. J. Comput. Appl. Math., **20:** 53-65.
[54] W.M. Rand **(1971)**. Objective criteria for the evaluation of clustering methods. J. Am. Stat. Assoc., **66:** 846–850.