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Epidemiology and Molecular Confirmation of *E. coli* Isolated from Diseased Fish in Muzaffargarh, Punjab, Pakistan

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Epidemiology and Molecular Confirmation of *E. coli* Isolated from Diseased Fish in Muzaffargarh, Punjab, Pakistan

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ABSTRACT

Background Fish is an important source of protein and vitamins, such as vitamin D and B2 (riboflavin) for human beings. However, they are plagued with a variety of disease-causing pathogens, resulting in significant economic losses. Among these pathogens, *Escherichia (E.) coli* are prominent, worldwide. This study aimed to conduct epidemiological surveillance and identification of *E. coli* strains isolated from diseased fish in District Muzaffargarh, Punjab, Pakistan.

Methodology A total of fifty (50) diseased fish samples were collected from various fish farms in the district. The isolation process involved enriching the samples in nutrient broth and incubating them at 37°C for 24 hours. After enrichment, the samples were inoculated on MacConkey agar and incubated again at 37°C for 24 hours. Following incubation, Gram staining was performed to identify *E. coli* and confirm its presence. These isolates were subjected to PCR using the *usp*A gene for confirmation.

Results Among fish diseases, Hemorrhagic septicemia was reported to have the highest prevalence (22%), while 12% of fish samples were infected with abdominal dropsy and fin rot. In total, six (06) *E. coli* isolates were obtained from five different diseased fish samples and confirmed by PCR-based detection of *usp*A gene.

Conclusion The current study found a link between disease-affected fish and naturally occurring *E. coli*, with molecular confirmation using the *usp*A gene. Effective management of soil, stock, water, nutrition, and environment is crucial to control losses caused by *E. coli* as opportunistic fish pathogens and spoilage agents.

Keywords: *Escherichia coli*, foodborne infections, food spoilage, opportunistic pathogens, *usp*A gene

Highlights

- This study confirmed the prevalence of *E. coli* in farmed fish based on molecular assay.
- Hemorrhagic septicemia was the most prevalent disease (22%) in the fish of the study area, followed by abdominal dropsy and fin rot (12%).
- Six (06) *E. coli* isolates were found among the fish infected with dropsy, ulcer, red spot, and hemorrhage septicemia infections.

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1. INTRODUCTION

Fish is an essential source of nutrition, globally. It provides 60% of protein supply and 16% of animal protein for human beings [1]. Fish is high in nutrients, such as omega-3 fatty acids and vitamins and is easily digestible, which is necessary for the growth and development of the body [2]. Pakistan is an agricultural state with both fresh water and marine resources. Aquaculture production is about 179,900 metric tons and from the natural catch it is 600,000 metric tons, contributing 1% to the GDP of Pakistan [3, 4]. Novel techniques are being developed in fish farming to fulfill nutritional requirements due to the increase in population [2]. Small-scale fish culture is also a source of income that helps to reducepoverty levels [5]. The aquaculture industry is expanding but infection rates are likely to rise, posing a significant risk to fish trade (lowering production rates and increasing fish mortality), human health (spreading diseases), and financial losses (lowering income and meat quality). Indeed, disease is a greater source of economic losses than any other factor [6]. Fish is affected by various kinds of bacterial, viral, and fungal diseases and various bacterial pathogens of fish are saprophytic [7]. Fish is a perishable food that becomes contaminated by improper handling and storage. So, fish and fish products management is a prime responsibility in the fish culture system to ensure public health.

The biggest cause of fish mortality are bacterial diseases in both natural and artificial culture systems [8, 9]. Bacterial pathogens are of two types, namely indigenous and non-indigenous pathogens. Non-indigenous pathogens, such as *Clostridium (C.) botulinum*, *Escherichia (E.) coli*, *Shigella (Sh.) dysenteriae*, *Staphylococcus (St.) aureus*, *Listeria (L)* and *Salmonella (Sa)* cause the contamination of fish and their habitats, whereas indigenous pathogens, such as Vibrio and Aeromonas species live naturally in the fish habitat [10].

E. coli is a harmful bacterium found naturally in the stomach and intestine of fish causes sickness [11, 12]. *E. coli* is a microorganism that is a Gram-negative, facultative, and anaerobic bacterium, belonging to the family *Enterobacteriaceae* of the class Gamma Proteobacteria [13]. *E. coli* can survive outside the body for a long period, making it ideal for food contamination. It is considered as a harmless intestinal pathogen; however, it is opportunistic and some of its strains have been identified as serious agents of severe illnesses [14, 15]. In local areas, health hazards linked with *E. coli* have become complicated because some causal strains have developed resistance against commonly used antibiotics [16].

Pathogenic *E. coli* causes foodborne diseases and contaminates fish and its products. Recently, food-related diseases have emerged as the most common issue causing serious health problems [17]. In Pakistan, food-related diseases are a significant cause of illness, with various major food-borne issues and their causes leading to infections that can vary from one disease to another. However, the fundamental sources of foodborne disease transmission in the country stem from unhygienic conditions, such as the absence of food standards, poor sanitation, poverty, and illiteracy, exacerbated by a lack of awareness [18]. Among these diseases, *E. coli* is a particularly concerning pathogen as it can cause severe infections in human beings, including diarrheal disease, life-threatening Hemorrhagic Colitis (HC), Hemolytic Uremic Syndrome (HUS), and Urinary Tract Infection (UTI), all of which

have been reported in Pakistan [19, 20, 21]. Moreover, the prevalence of *E. coli* has been reported in food items, water, soil, salad vegetables, and fruits [22, 23]. Fish is an important food source but no study is available about the prevalence of *E. coli* in fish in Pakistan. The current study aims to determine the prevalence of diseases and identification of *E. coli* strains from diseased fish.

Figure 1. Map of the Study Area Showing Potential Sampling Sites (Arc GIS software, 2020)

2. MATERIALS AND METHODS

2.1. Study Area

The current study was performed in different fish farms of District Muzaffargarh, Punjab, Pakistan. Muzaffargarh lies between 30° 4' 27.7572" N and 71° 11' 4.7544" E. It is subdivided into four tehsils, namely Muzaffargarh, Alipur, Kot Adu, and Jatoi. The sampling area is shown in Figure 1.

It is the hub of fish hatcheries, namely Chenab Fish Hatchery and Tawakkal Fish Hatchery, fish farming, and agriculture. Rahu *(Labeo rohita)* and Tilapia *(Oreochromis niloticus)* are the most culturable fish species in this region. The nature and quantity of various salts in the soil and water of District Muzaffargarh are best suited for fish culture. Rahu Rangla Wetland Complex is located in this district

and covers an area of 24,140 hectares. It is a combination of many small and medium-sized lakes. All these lakes are present in a 25 to 30 km area. Twenty-four (24) fish species have been reported from the Rangla Wetland Complex [24].

2.2. Data and Sample Collection

Random sampling was done in the four tehsils of District Muzaffargarh. Fifty (50) fish samples of Rahu (*Labeo rohita* =19), Mori (*Cirrhinus cirrhosus*=3), Grass carp *(Ctenopharyngodon idella*=12*)*, Silver carp *(Hypophthalmichthys molitrix*=4*)*, Tilapia *(Oreochromis niloticus*=10*)*, Catfish *(Siluriformes*=1*)*, and Gulfam *(Cyprinus carpio*=1*)* with signs of dropsy, ulcer, fin rot, hemorrhage septicemia, scale damage, lernea, and skin lesions were randomly collected from different fish farms in each of these districts. The samples were kept in a sterile zip-lock bag and placed in an icebox filled with crushed ice. Then, these samples were transported to Epidemiology and Microbiology Laboratory, Department of Wildlife and Ecology, the University of Veterinary and Animal Sciences, Pattoki.

2.3. Dissection and Collection of Fish Organs

First, the fish were cleaned with 70% alcohol. Then, fish species, body weight, body width, and body length were noted. Afterward, the fish were dissected to collect different organs (liver, muscles, spleen, and heart) in order to isolate the bacteria [25]. To isolate bacteria from fish skin, sterilized cotton swabs were rubbed on it and then it was incubated in nutrient broth for enrichment for 48 hours at 37°C. To isolate bacteria from gills, kidneys, and liver, these organs were removed with the help of sterile forceps and enriched in nutrient broth for 24 hours at 37°C.

2.4. Isolation and Characterization of *E. coli*

Initially, the enrichment of fish samples (muscles, kidneys, spleen, and liver) was performed by incubation in nutrient broth (Lab M Ltd. United Kingdom) at 37°C for 24 hours. After enrichment, all samples were inoculated on an *E. coli* isolation media MacConkey agar (Himedia Laboratories Pvt. Ltd, Mumbai, India) and incubated at 37°C for 24 hours. After 24 hours of incubation, the isolates were selected based on colonial morphology having smooth, circular, moist, and dark pink colonies. All isolates of *E. coli* were confirmed using Gram staining (rod shape and pink). Afterward, selected colonies were grown on Eosin Methylene Blue (EMB) agar. Colonies with a green metallic sheen and dark center on EMB were predicted as *E. coli*. To purify the isolated colonies, they were subcultured on Tryptic Soy Agar (TSA) plates from Lab M Ltd. in the United Kingdom. Subsequently, the *E. coli* isolates were preserved using a 50% glycerol solution [26].

2.5. DNA Extraction of *E. coli*

The boiling method was used for DNA extraction. In 100 µl distilled water, a single colony of positive culture was introduced and boiled for 15 minutes at 100°C, then treated with 5 minutes of centrifugation at 10,000 rpm. The supernatant was carefully collected and stored at -4°C in a separate Eppendorf [27].

2.6. Molecular Amplification of *E. coli usp*A gene

The presence of *E. coli* was confirmed by amplifying the *usp*A (884bp) gene, which codes for the universal stress protein, following the previously described method [28]. A reaction mixture of 25 µl was

prepared, consisting of 12 µl of master mix, 2.5 µl of template DNA, and 1 µl each of forward A (F-5'-CCGATACGCTGCCAATCAGT-3') and reverse primers (R-5'-ACGCAGACCGTAAGGGCCAGAT-3'), with 8.5 µl of injection water. The amplification was carried out using a BioRad T100 Thermal Cycler with a cycling program comprising 30 cycles with denaturation at 94°C for 5 minutes, annealing at 55°C for 10 seconds, extension at 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. The resulting amplified DNA (5 µl per lane) was then separated and visualized on a 2% agarose gel at a constant voltage of 100 V, using UV light on a BioRad Gel Doc TM EZ Imager.

2.7. Data Analysis

A bar graph was employed for visual representation in the context of a chi-square analysis aimed at examining the prevalence of disease among culturable fish species.

3. RESULTS

A total of 50 diseased fish samples were collected for the prevalence and characterization of *E. coli*. Epidemiological data of the collected fish samples is given in Table 1. Multiple diseases were observed in fish species. Among these, 9 (18%), 11 (22%), 9 (18%), 6 (12%), 1 (2%), 2 (4%), 2 (4%), 2 (4%), 2 (4%), 1 (2%), and 5 (10%) species were infected with dropsy, hemorraghe septicemia, fin rot, lernea, scale damage, red spot, wound, fungus, deformation, decoloration, and ulcer, respectively (Table 2). A graphical representation of multiple disease prevalence in fish species is shown in Figure 2. Out of 50 fish samples, 19 (38%), 3 (6%), 12 (24%), 4 (8%), 10 (20%), 1 (2%), and 1 (2%) were Rahu (*Labeo rohita*), Mori (*Cirrhinus cirrhosus*), Grass carp *(Ctenopharyngodon idella)*, Silver carp *(Hypophthalmichthys molitrix)*, Tilapia *(Oreochromis niloticus)*, Catfish *(Siluriformes)*, and Gulfam *(Cyprinus carpio)*, respectively (Table 3). A total of 5 out of 50 infected fishes were contaminated by *E. coli*. A total of 6 isolates of *E. coli* were obtained from 5 different diseased fish. Among these 6 isolates, 2 (33.3%) were obtained from the epidermal muscle and intestine of Rahu (*Labeo rohita).* The other 4 (66.6%) isolates of smooth, circular, moist, and dark pink colonies were isolated from the epidermal muscle of Grass carp *(Ctenopharyngodon idella)*, Silver carp *(Hypophthalmichthys molitrix)*, Gulfam *(Cyprinus carpio)*, and Mori (*Cirrhinus cirrhosus*) (Table 4). All 6 (12%) isolates from diseased fish were found positive through PCR by amplifying the *usp*A gene of *E. coli* (Figure 3).

Table 1. Epidemiological Data Collected from Diseased Fish Samples from District Muzaffargarh

Sample no.	Fish Species	Disease	Total length (cm)	Weight (g/kg)
1.	*Labeo rohita*	Hemorrhage Septicemia	16	930
2.	*Ctenopharyngodon Idella*	Ulcer	11.5	224
3.	*Hypophthalmichthys molitrix*	Dropsy	6.5	52
4.	*Cirrhinus cirrhosis*	Dropsy	32	562
5.	*Ctenopharyngodon Idella*	Fin Rot	42	922

Sample no.	Fish Species	Disease	Total length (cm)	Weight (g/kg)
6.	*Labeo rohita*	Fin Rot	42	1250
7.	*Labeo rohita*	Deformation	43	1682
8.	*Ctenopharyngodon Idella*	Fin Rot	37	946
9.	*Labeo rohita*	Fin Rot	38	938
10.	*Ctenopharyngodon Idella*	Scale Damage	43	1426
11.	*Hypophthalmichthys molitrix*	Deformation	38	762
12.	*Labeo rohita*	Hemorrhage Septicemia	34	648
13.	*Hypophthalmichthys molitrix*	Red Spots	39	710
14.	*Labeo rohita*	Wounds	33.4	504
15.	*Ctenopharyngodon Idella*	Wounds	36	568
16.	*Ctenopharyngodon Idella*	Red Spots	46.5	1496
17.	*Ctenopharyngodon Idella*	Ulcer	42	794
18.	*Ctenopharyngodon Idella*	Fin Rot	40	680
19.	*Ctenopharyngodon Idella*	Decoloration	45	900
20.	*Labeo rohita*	Ulcer	47	1342
21.	*Labeo rohita*	Ulcer	39	558
22.	*Labeo rohita*	Dropsy	12.5	134
23.	*Oreochromis niloticus*	Hemorrhage Septicemia	12	36
24.	*Oreochromis niloticus*	Hemorrhage Septicemia	12.5	36
25.	*Oreochromis niloticus*	Hemorrhage Septicemia	8	38
26.	*Oreochromis niloticus*	Dropsy	15	17
27.	*Siluriformes*	Fin Rot	14	490
28.	*Labeo rohita*	Lernaea	9	628
29.	*Oreochromis niloticus*	Hemorrhage Septicemia	8	248
30.	*Cyprinus carpio*	Dropsy	13	60
31.	*Labeo rohita*	Lernaea	10	416
32.	*Labeo rohita*	Lernaea	8	228
33.	*Cirrhinus cirrhosis*	Dropsy	8.5	66
34.	*Labeo rohita*	Lernaea	12	114

Sample no.	Fish Species	Disease	Total length (cm)	Weight (g/kg)
35.	*Oreochromis niloticus*	Fin Rot	9.5	644
36.	*Labeo rohita*	Hemorrhage Septicemia	10.3	157
37.	*Oreochromis niloticus*	Dropsy	13	490
38.	*Ctenopharyngodon Idella*	Fin Rot	9	648
39.	*Oreochromis niloticus*	Dropsy	11.5	292
40.	*Oreochromis niloticus*	Fin Rot	8	574
41.	*Oreochromis niloticus*	Dropsy	37.5	175
42.	*Hypophthalmichthys molitrix*	Hemorrhage Septicemia	35	464
43.	*Labeo rohita*	Lernaea	50	460
44.	*Labeo rohita*	Fungus	22	1478
45.	*Labeo rohita*	Lernaea	30	126
46.	*Ctenopharyngodon Idella*	Hemorrhage Septicemia	36	391
47.	*Labeo rohita*	Fungus	19	990
48.	*Labeo rohita*	Fin rot	17	74
49.	*Ctenopharyngodon Idella*	Hemorrhage Septicemia	23	64
50.	*Cirrhinus cirrhosis*	Hemorrhage Septicemia	20	112

Table 2. Prevalence of Various Fish Diseases in Collected Samples

Sample No	Disease Types	No	Percentage (%)
1.	Hemorrhage Septicemia	11	22
2.	Dropsy	9	18
3.	Fin rot	9	18
4.	Lernaea	6	12
5.	Scale damage	1	2
6.	Red spot	2	4
7.	Wound	2	4
8.	Fungus	2	4
9.	Deformation	2	4
10.	Decoloration	1	2
11.	Ulcer	5	10
Total	-	50	-

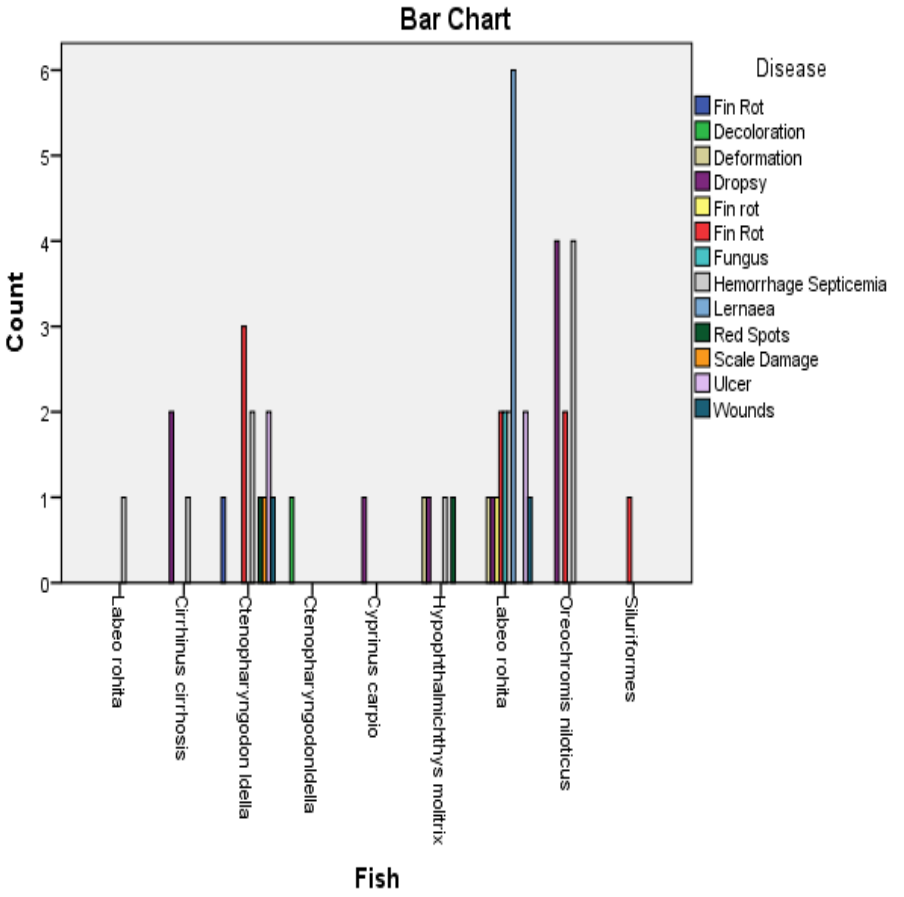

Figure 2. Bar Graph Showing Chi-square Analysis of Disease Prevalence in Culturable Fish Species

Table 3. Frequency and Percentage of Collected Fish Species

Sample No	Fish types	Total collected	Percentage (%)
1.	*Cirrhinus cirrhosis*	3	6
2.	*Ctenopharyngodon idella*	12	24
3.	*Labeo rohita*	19	38
4.	*Hypophthalmichthys molitrix*	4	8
5.	*Oreochromis niloticus*	10	20
6.	*Siluriformes*	1	2
7.	*Cyprinus carpio*	1	2
Total count.	-	50	-

Table 4. Details of Culture-Positive Fish Samples

Sample No	Sample Type	Fish	Disease	Isolated Species
34	Epidermal Muscle Intestine Kidney	*Labeo rohita*	Lernaea	*E. coli*
36	Epidermal Muscle Intestine Kidney Liver	*Labeo rohita*	Hemorrhage Septicemia	*E. coli*
18	Epidermal Muscle Spleen Kidney	*Ctenopharyngodon Idella*	Ulcer	*E. coli*
13	Epidermal Muscle Spleen Intestine	*Hypophthalmichthys molitrix*	Red spot	*E. coli*
30	Epidermal Muscle Kidney Liver	*Cyprinus carpio*	Dropsy	*E. coli*
33	Epidermal Muscle Intestine Kidney Spleen	*Cirrhinus cirrhosis*	Dropsy	*E. coli*
Total. 6	-	6	-	-

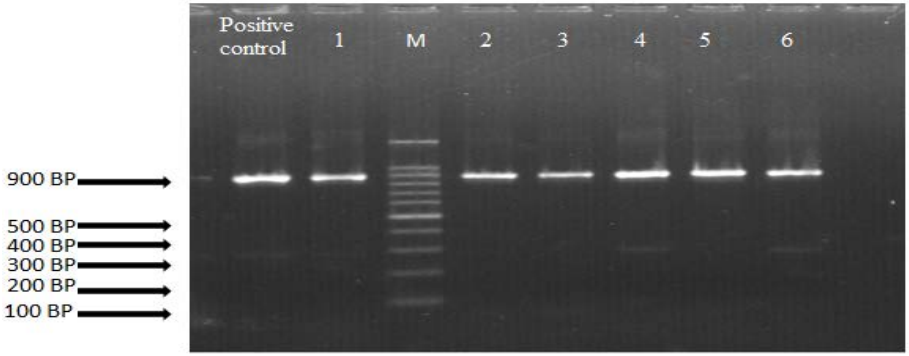

Figure 3. PCR-based Identification of *E. coli* on 1.5% Ethidium Bromide-stained Agarose Gel, Lane M: (left to right) 100bp ladder (GeneOn), Lane 1-6 Confirmed Field Isolates of *E. coli* (*usp*A gene) (884 bp) Primer Amplification

4. DISCUSSION

E. coli is a widespread pathogenic bacterium found in fish meat and fishpond water [29]. The fish industry is highly affected by *E. coli* which causes food poisoning and spoilage [30, 31]. The current study provides us the evidence of the presence of *E. coli* in diseased fish from different fish farms of District Muzaffargarh, Punjab, Pakistan. There is a dire need for incorporating control strategies of *E. coli* in Pakistani aquaculture.

In the current study, 50 diseased fish samples were collected from different fish farms of District Muzaffargarh. *E. coli* were isolated from 6 samples of various fish species including 2 from Rohu (*Labeo rohita*), 1 from Grass carp (*Ctenopharyngodon idella)*, 1 from Mori (*Cirrhinus cirrhosus)*, 1 from Silver carp *(Hypophthalmichthys molitrix)*, and 1 from Gulfam *(Cyprinus carpio).* A similar study was conducted in Tanzania to assess the bacterial flora responsible for the spoilage of fish, with *E. coli* prevalence observed in the fish as high (39%) [32]. In the current study, fish species were found to be infected with various bacterial diseases including hemorrhage septicemia, dropsy, fin rot, and lernaea. Similarly, in a previous study, fish from a freshwater fish farm in Egypt was found to be affected with hemorrhage, gill rot, and red spot disease [33]. In the current study, the prevalence of hemorrhage septicemia (22%), dropsy (18%), fin rot (18%), lernaea (12%), scale damage (2%), red spot (4%), wound (4%), fungus (4%), deformation (4%), decoloration (2%), and ulcer (10%) was reported. Similar findings were reported in Assam, India where a high prevalence of ulcerative syndrome (28.01%) and red spot/hemorrhages (18.82%) was found in different farmed fishes [34]. In the current work, out of 50 fish samples, 19 (38%), 3 (6%), 12 (24%), 4 (8%), 10 (20%), 2 (1%), and 2 (1%) were Rahu (*Labeo rohita*), Mori (*Cirrhinus cirrhosus*), Grass carp *(Ctenopharyngodon Idella)*, Silver carp *(Hypophthalmichthys molitrix)*, Tilapia *(Oreochromis niloticus)*, Catfish *(Siluriformes)*, and Gulfam *(Cyprinus carpio).*

Previously, according to a study conducted in Pakistan, 60 fish were collected from fishponds including *Labeo rohita* (Rohu*)*, *Catla catla (Thaila)*, and *Cirrhinus mrigala (Morakhi)* for bacterial confirmation. It was found that *E.coli* was the most prevalent bacteria in pond fish with an occurrence rate of 86.6% [35]. In the current study, a total of 6 isolates of *E. coli* were obtained from 6 different diseased fish. Among these 6 isolates, 2 were obtained from the epidermal muscle and intestine of Rahu (*Labeo rohita*). The other 4 isolates were isolated from the epidermal tissue of Mori (*Cirrhinus cirrhosus*), Grass carp *(Ctenopharyngodon Idella)*, Silver carp *(Hypophthalmichthys molitrix)* and Gulfam *(Cyprinus carpio)*, respectively. All these 6 isolates were positive for *E. coli*. In a previous study in Ethiopia, 5 isolates of *E. coli* were isolated from the intestine, 2 from the kidneys, and 5 from the liver [36]. In the current study, 6 (12%) fish samples were found to be infected with *E. coli*. However, the higher isolates of *E. coli* were obtained from the intestine and muscle. while, the liver, kidneys, and spleen exhibited a lower level of isolates.

Previously, *E. coli* was first discovered in the intestine of wild fish [11]. In the current study, 12% of isolates of *E. coli* were recorded from diseased fish. This relates to a former study [37] which recorded 33.3% of lake fish sample containing *E. coli*. *E. coli* prevalence was found to be 12% in the current study, as compared to 8.9% in

earlier studies [38]. However, in other previous studies, a higher prevalence (23.2%) was reported [39]. *E. coli* is a part of the normal intestinal microflora of fish that is more frequently isolated from its intestine than from its spleen and liver [37]. Intestinal microbiota can cause health problems if they spread to other organs or are linked to product contamination during fish processing, which has serious implications for both fish and human health [40]. In the current study, all 6 (12%) isolates from diseased fish were found to be positive through PCR by amplifying *usp*A gene of *E. coli*. Similarly, in a previous study, 10% of *E. coli* isolates from 10 frozen fish samples that were collected from different markets of Basrah, Iraq were found to be positive through PCR by amplifying *usp*A gene of *E. coli* [41]. In another study, 13% of *E. coli* isolates from Indian foods comprising marine fishes, mutton, beef, and pork out of 43 (22%) samples were positive by amplifying the *usp*A gene [26].

4.1. Conclusion

E. coli is the most important pathogenic bacteria that causes severe economic losses in the fish industry and contaminates fish food. Smallholder farmers who depend upon the fisheries sector for their annual income are directly affected by such losses. Further study should be conducted to find out its possible control strategies and fish should be processed under hygienic conditions to control intestinal content contamination.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVALIABILITY STATEMENT

The data associated with this study will be provided by the corresponding author upon request.

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