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RESEARCH ARTICLE

Prevalence of the *copA* Gene in *Escherichia coli* Isolated from Common Carp in Sulaymaniyah Province

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The presence of pathogenic *Escherichia coli* in human consumption of food fish can cause disease and might increase the likelihood of antibiotic resistance transmission from aquatic bacteria to bacteria that infect humans. The study aimed to detect the copper resistance gene (*copA*) in the isolated *E. coli*. from the common carp fish of Dukan Lake in Sulaymaniyah province. Ninety fish samples were taken from three fishing places at Dukan Lake; the center of Dukan Lake, Ranya, and Qaladze. Isolation and identification of *E. coli* were performed using culturing and molecular methods. Of the 90 fish samples, 58 (64.4%) were positive for *E. coli.* The isolates were screened for detection of the 16S rRNA and the *copA* genes by Polymerase Chain Reaction (PCR) using specific primers. Of the 90 fish samples, 46 (51.1%) were positive for the 16S rRNA gene, and 6 (6.7%) were positive for the *copA* gene. The *E. coli* gene 16S rRNA has been sequenced and assigned accession number ON385541 under the name of *E. coli* isolate 2022 mabao in NCBI GenBank. The phylogenetic analysis of 16S rRNA gene sequences revealed that *E. coli* strains were related to other *E. coli* bacteria from other countries, with the maximum identity being 99.61 percent. We conclude that small amounts of *E. coli* found in common carp in three distinct areas of Dukan Lake have the *copA* gene, and an investigation of the 16S rRNA gene sequence in our study revealed that this gene's conserved region only had a few small nucleotide alterations.

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INTRODUCTION

Proteins, minerals, vitamins and polyunsaturated fatty acids are all abundant in fish. Fish is tasty, incredibly nutritious and quickly absorbed (Kumar *et al.,* 2018; Bulut and Rashid, 2020). *Escherichia coli* is a marker biotic used to track fecal contamination of aquatic and sea-meat (Avşar and Berber, 2014; Terzi, 2018). *E. coli* belongs to the *Enterobacteriaceae* family, that can grow at temperatures between 7°C and 45°C with a respiratory and fermentative metabolism and oxidase negative (Garbaj *et al.,* 2016).

There are many publications on the increased awareness of heavy metal contamination of aquatic ecosystems (Bawuro *et al.,* 2018). Heavy metal bioaccumulation in the body's tissues of various aquatic organisms produces several adverse effects; it can have cytotoxic, immunosuppressive, mutagenic, and genotoxic effects (Salamat *et al.,* 2015; Matos *et al.,* 2017). After consuming contaminated fish, heavy metals deposited on aquatic organisms can enter the human body and have an adverse influence on human health (Abarshi *et al.,* 2017).

It is also known that *E. coli* carries genes for many resistances, including antibiotic, disinfectant, and heavy metal genes. Bacteria can adapt their genetic structure and physiological composition to survive against heavy metals that are present in soils, plants, and aquatic systems. As a result, in reaction to these contaminants, the bacteria may produce heavy metal resistance genes (HMRGs). The HMRG can be detect on bacterial plasmids as well as chromosomes (Mol *et al.,* 2017). Bacteria in fish, like *E. coli*, may possibly develop resistance genes to particular heavy metals when specific metals, like copper, accumulate to a threshold level, a very low amount of metals was recently shown in various papers (far lower than the minimum inhibitory concentration (MIC)) enabled bacteria to acquire a resistant plasmid that contains genes for antibiotic and heavy metal resistance through co-selection (Gullberg *et al.,* 2014; Chen *et al.,* 2015). The MIC for *E.*

coli is 100-400 µg/ml (Terzi and Civelek, 2021). More importantly, because these heavy metal ions left over from livestock farms may contaminate the environment. They can be used as bioindicators to identify environmental heavy metal pollution. (Seiler and Berendonk, 2012; Ozer *et al.*, 2013).

Isolation of *E. coli* can be done in a variety of ways. The decision is made based on the target strain and isolation aim. *E. coli* can be distinguished from other coliforms that do not ferment lactose using MacConkey's agar because of its ability to ferment lactose. Biochemical, enzymatic, or molecular techniques can be used to confirm the identification (Meiyarasi *et al.*, 2017). Specific nucleic acids can be identified using molecular diagnostic methods without the necessity for pathogen isolation or culturing. All prokaryotic organisms contain highly conserved portions of the 16S rRNA gene, and it also has variable areas that have been utilized to discriminate between species and isolates and to offer important taxonomic information at different levels of classification (Darwish *et al.*, 2004; Fattahi *et al.*, 2013).

According to previous studies, Dukan Lake as well as its water resources showed higher copper concentrations than normal among other heavy metals. Therefore, we carried out this study to investigate the *copA* gene in *E. coli* isolated from common carp in the Sulaymaniyah province.

MATERIALS AND METHODS

The region of study and sampling: From Dukan Lake, common carp samples were taken between the winter of 2021 and the spring of 2022. Around 76 kilometers from the city center, Dukan Lake is situated in the northwest of Sulaymaniyah, is regarded as Iraq's Kurdistan region's biggest lake. Numerous urban communities and cities are dispersed around the Dukan Lake boundary. Agriculture, animal breeding and some industrial activities are the primary activations in the study area. The lake has an operating full-pool altitude of 515 m above mean sea level and its boundaries are located between latitudes 34°17'N and 36°33'N and longitudes 43°17'E and 46°24'E. The drainage region covers roughly 11,690 km^2 in total, of which 1080 km^2 are on the plains of Qaladze and Ranya. The lake is a result of the Dukan dam, which was built in 1959 upstream of the same-named town. Rivers are nourished by rain and snowfall, which causes springtime peak discharge and summer and early fall low water. The lake itself is split into two portions by a curved valley that divides a larger lake to the north from a smaller lake to the south (Toma, 2013).

From three different sections of Dukan Lake, 90 fish samples were collected. Each of the fishing areas (center of Dukan Lake, Ranya, and Qaladze) received 30 samples (15 for winter and 15 for spring). The labeled and sterile plastic containers were used to put the samples and transported in cool boxes to the high education lab Veterinary Medicine college/Sulaimani University for isolation and identification of bacteria *E. coli* on the same day they were taken from the fishers.

***E. coli* isolation and identification:** All the fish samples from the three fishing areas had their intestines handled separately without using pooled samples. Each fish's intestine was extracted using disposable sterilized scissors and blades. After being aseptically cultured on MacConkey's broth (Neogen, UK) and allowed to incubate for 24 hours at 37°C. A loop full of the incubated broth was plated on the selective media, MacConkey's agar (Liofilchem, Italy) and Eosin Methylene Blue (EMB) agar **(**Liofilchem, Italy), then left to incubate at 37 °C for 24 h (Zhang *et al.*, 2016). Suspected *E. coli* (dark blue to purple colonies) were verified using DNA sequencing of the 16S rRNA gene.

Extraction of DNA: Typically, the boil and snap method of cooling was used for DNA extraction. 1.5 mL of a nutrient broth culture (Liofilchem, Italy) that had been cultivated overnight was put in sterile micro centrifuge tubes, and was centrifuged at 10,000 *g* for five minutes. The pellets were re-suspended in 1.0 mL of sterile distilled water after being rinsed twice with sterile distilled water. After 20 minutes in a boiling water bath, the tubes were immediately chilled on ice cubes for at least 20 minutes. After tubes had been centrifuged at 10000*g* for 2 minutes, 5µL of the clear supernatant layer was used as the template DNA for PCR tests (Gupta *et al.*, 2013). However, when this method did not yield the expected results, genomic DNA was isolated using a DNA extraction kit (South Korea) from overnight growth colonies on agar plates. The process was performed according to the manufacturer's instructions. DNA quality was assessed on a 1% agarose gel (Genedirex, USA) in 1x Tris/Borate/EDTA (TBE) buffer using loading combinations (6µL DNA and 2µL loading dye). After applying 10µL of safe stain (GeNet Bio, Korea) to the gel to stain it, electrophoresis was carried out at 85V for 50 minutes.

Oligonucleotides: A set of specific primer was used to distinguish *E. coli* from fish and detect the heavy metal resistance gene (*copA*). Regarding the 16S rRNA gene sequence, FES-F and RES-R primers were applied; the expected amplicon size is 544 bp (Fattahi *et al.*, 2013). For the *copA* gene, the copA-F and copA-R primers were used; the size of the amplicon is 1200 bp (Ture *et al.*, 2021). The target gene, primer name and sequence, amplified fragment, as well as annealing temperature are listed in Table 1. The primers were synthesized by Macrogen (South Korea).

PCR amplification: The AddStart Taq Master (PCR) (Korea) was used to amplifying the genes. We used 0.2 mL PCR tubes. The PCR tube was filled with 10µL a master mix, 5µL of DNA, and 1µL (10 pmol) of each forward and reverse primer. By adding 3µL of DEPC-treated water, the full amount of 20µL was attained.

The thermal cycler program (Prime, UK) started with an initial denaturation step at 94°C for 7 min, followed by 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 63.7°C (16S rRNA) and 55°C (*copA* gene) for 1 min, 72°C for 1 min extension), and a final 10 min extension period at 72°C.

After that, 7µL of PCR products were loaded onto a 1% agarose gel (Genedirex, USA) in 1 Tris/Borate/EDTA (TBE) buffer, and the PCR product was analyzed. A 5µL safe dye (GeNet Bio, Korea) was used to stain the gel; electrophoresis was performed for 45 min at 120 volts.

After confirmation of the 16S rRNA gene was done by comparing with the 100 bp DNA ladder (GeneDirex**)**, the positive PCR results were confirmed by sequencing the amplified DNA products.

Sequencing of the 16S rRNA gene and phylogenetic analysis: The PCR results were sequenced using the Sanger sequencing method (Macrogen, Korea). Following that, using the Clustal W tool, the results were aligned with the *E. coli* 16S ribosomal RNA reference sequences available in NCBI GenBank. Subsequently, the neighbor-joining method and evolutionary analyses were conducted in MEGA X (Saitou and Nei, 1987; Kumar *et al.,* 2018). The tarmura3-parameter model with 1000 bootstrap replicates was used to determine phylogenetic relationships (Tamura, 1992).

RESULTS

Samples: In this study, 90 intestinal samples were collected from common carp in three distinct areas of Dukan Lake. Fifty-eight samples (64.4%) from common carp were found positive for *E. coli*. A high percentage of the bacteria (83.3%) isolated was especially prevalent in the Ranya area (Table 2). On MacConkey agar, the isolated *E. coli* showed up as pinkish colonies, while on Eosin Methylene Blue, they had a green metallic sheen.

Identification of 16S rRNA & *copA* genes: In the present study, *E. coli* DNA was successfully extracted from the colonies on Eosin Methylene Blue agar. For the 16S rRNA gene, 46 isolates (51.1%) from fish were found to be positive. In the Ranya area, a large percentage of the bacteria (66.7%) isolates were positively identified for the 16S rRNA gene. A total of 6 (6.7%) isolates were positive for the presence of the *copA* gene. The *copA* gene is not detected in the isolates from fish in the center of the Dukan area (Table 2).

As shown in Fig. 1, agarose gel electrophoresis showed that *E. coli* in the current study was positive for the 16S rRNA gene, showing a 544 bp amplicon and the identification of the *copA* gene in *E. coli* isolated from fish with a 1200 bp amplicon.

Sequence analysis: With the accession number (ON385541), the PCR result from one of the positive samples was submitted to the International Nucleotide Sequence Database (INSD), a part of the National Center for Biotechnology Information (NCBI). A subset of the 16S rRNA gene sequences from the field strains and representative strains from GenBank was analyzed using phylogenetic analysis to evaluate and verify the evolutionary lineage of the field samples. Results showed that the field strain was related to other *E. coli* strains from various countries (Algeria-MW480214, Vietnam-OM943787, and India-ON287271) with the highest identity of 99.61% (Fig. 2).

DISCUSSION

Bacteria can adapt their genetic structure and physiological composition to survive against heavy metals that are present in aquatic systems. As a result, the bacteria may develop and harbor heavy metal resistance genes. The *copA* gene is associated with copper resistance in bacteria (Mol *et al.,* 2017). In agriculture and aquaculture, copper has been used as a growth booster and as a preventative measure for bacterial and parasitic fish diseases (Hobman and Crossman, 2015). Heavy metals are absorbed from the water by fish through their gills, digestive tracts, and skin. Some of the heavy metals are distributed and accumulate in various tissues with blood circulation, while others are eliminated via urine and feces. In aquatic life, fish represent the last link in the food chain; heavy metals can then reach humans after passing through fish meat. Hence, it's possible to find copper in few breeds of demersal fish (Arantes *et al.,* 2016).

In the present study, we started by searching for an isolate of *E. coli* from common carp in the Dukan Lake. The study revealed that 58 out of 90 intestinal samples from common carp tested positive for *E. coli* (Table 2). Our results agreed with Ameer (2016) since *E. coli* was isolated from samples of common carp in Baghdad. On the other hand, Meiyarasi *et al.,* (2017) identified *E. coli* from fish intestines in India. Also, Austin and Austin (2016) initially found *E. coli* in the intestines of wild fish. Many fish are frequently exposed to different bacteria. These microorganisms are common in the environment and some get into water from human waste, agricultural effluent, and animal feces. Similar to *E. coli*, large numbers of bacteria can linger in the skin and intestines of fish, potentially endangering public health (Costa, 2013).

The 16S rRNA gene was examined using PCR to verify the identification of *E. coli*. Forty-six (51.1%) of the tested samples were positive for the 16S rRNA gene (Table 2). A similar result was reported by Fattahi *et al.* (2013) as they targeted the 16S rRNA gene for the identification of *E. coli* from Iranian fish by using the same set of primers (FES, RES). The 16S rRNA gene area was amplified using a primer in samples from fish cases, and the results show that this region is more reliable for the identification of *E. coli*. As shown in Fig. 2, the field sequence showed a high degree of similarity (99.61%) and was closely linked to those strains according to the phylogenetic tree and sequence comparison of the 16S rRNA genes. Only one cluster cannot be isolated from the evolutionary tree because the 16S rRNA gene conserves this area. With the exception of sporadic instances, nucleotide substitutes are similarly avoided in this region.

The investigation of the *copA* gene in *E. coli* isolated from common carp in the Dukan Lake in the province of Sulaymaniyah is the major goal of the study. The *copA* gene, which encodes a P-type copper-transporting ATPase, has been discovered to be present in a variety of gram-positive bacteria, such as *Lactococcus garvieae*, and gram-negative bacteria, such as *Acinetobacter baumannii, E. coli*, *Enterococcus faecium*, and *Salmonella* spp. (Yang *et al.,* 2020). In our study small amounts of the *copA* gene were found in *E. coli* (6.7%) isolated from fish. This result is lower than the finding of Ture *et al.* (2021), who reported that the most prevalent metal resistance genes in bacteria isolated from fish in the southeast Black Sea are (46.2, 35.8% and 17.9%) for *copA*, cadmium-zinc-cobalt (*czc*) and nickel-cobalt-cadmium (*ncc*) respectively, the *copA* gene abundance is influenced by the physicochemical properties of the aquatic environment (Ture *et al.,* 2018).

Table 1: Primers used in the PCR.

Target gene	Primer	Sequence (5'–3')	Length of an amplified segment (bp)	Annealing temperature (°C)	Reference
16S rRNA	FES-F	GGAAGAAGCTTGCTTCTTTGCTG			
	RES-R	AGCCCGGGGATTTCACATCTGA	544	63.7	Fattahi *et al.,* 2013
copA	copA-F	GGSASDTACTGGTRBCAC	1200	55	Ture *et al.,* 2021
	copA-R	TGNGHCATCATSGTRTCRTT			

Table 2: Results from PCR testing and the prevalence of *E. coli* found in common carp.

District name	Samples tested	Number of *E. coli* isolates (%) (On EMB agar)		PCR results			
				Number of *E. coli* isolates (%) (For 16S rRNA gene)		Number of *copA* gene (%)	
		Positive	Negative	Positive	Negative	Positive	Negative
Center of Dukan	30	16 (53.3)	14 (46.7)	16 (53.3)	14 (46.7)	0.0	30 (100)
Ranya	30	25 (83. 3)	5 (16.7)	20 (66.7)	10 (33.3)	4 (13.3)	26 (86.7)
Qaladze	30	17 (56.7)	13 (43.3)	10 (33.3)	20 (66.7)	2 (6.7)	28 (93.3)
Total	90	58 (64.4)	32 (35.6)	46 (51.1)	44 (48.9)	6 (6.7%)	84 (93.3)

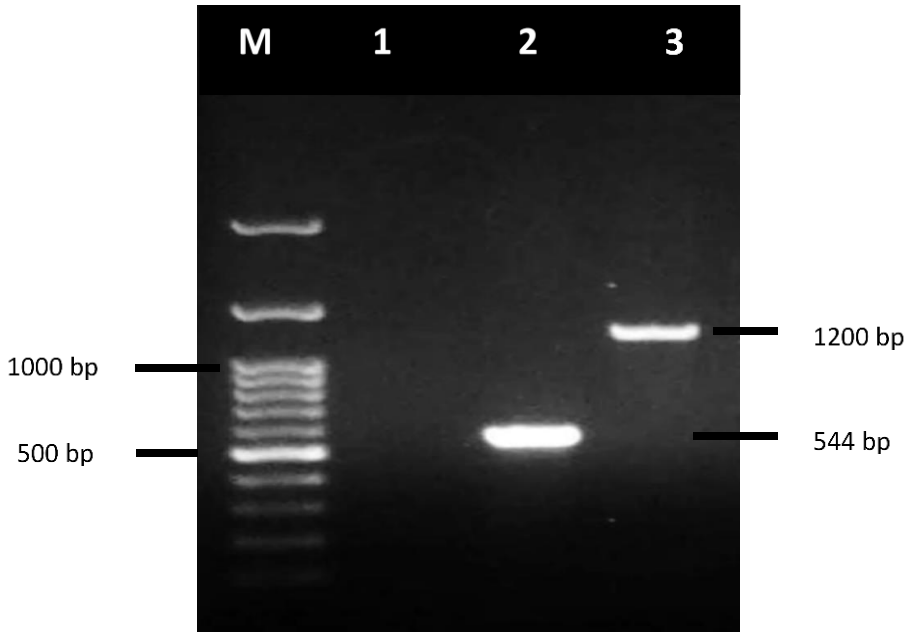

Fig. 1: Target DNA from *E. coli* is specifically amplified by PCR using particular primers. Lane M: Display the 100 bp DNA size marker, lane 1: Negative control (PCR reaction mix without DNA), and lane 2: Sample of the 16S rRNA gene (544bp) and lane3: *copA* gene (1200bp).

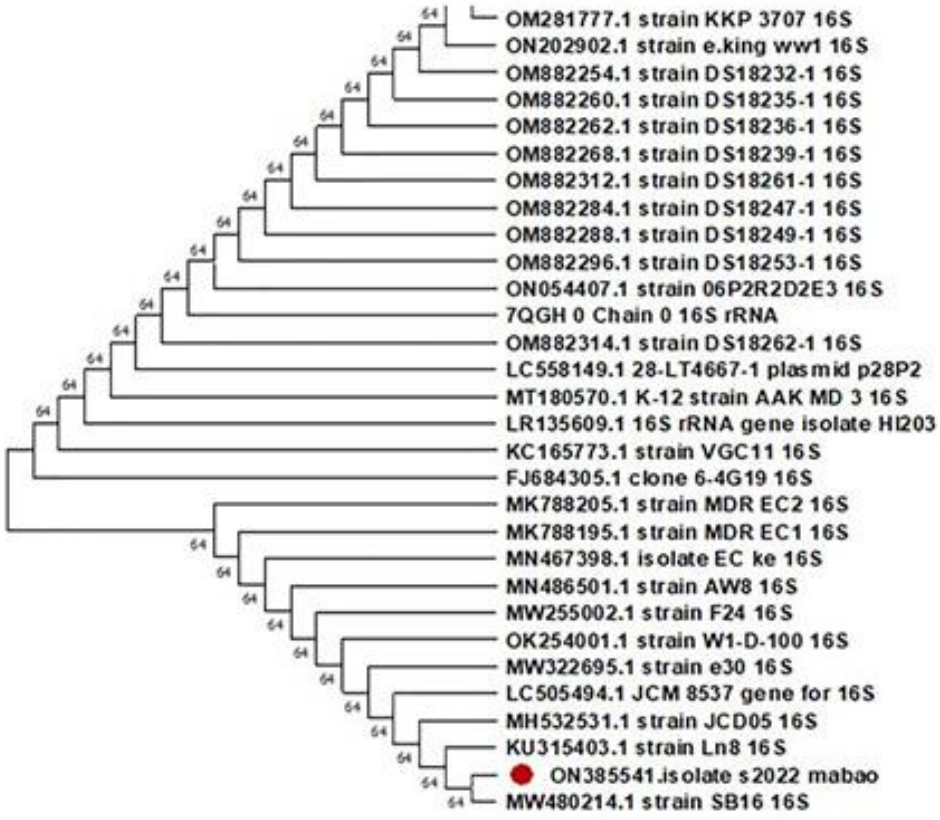

Fig. 2: Phylogenetic tree generated based 16S ribosomal RNA sequence data of *E. coli* spp. in this study and similar sequences from GenBank database constructed using Neighbor-joining method, MEGA X.

Resistance genes can be transferred or exchanged between bacteria from several sources, including humans, animals, and soil. Several studies have documented the existence of resistance genes in bacteria that confer tolerance to contaminants such as heavy metals (Matyar, 2012; Ture *et al.*, 2021).

Conclusions: The research's findings, which also highlighted the meager amounts of *E. coli* found in common carp in three distinct areas of Dukan Lake, have the *copA* gene. Furthermore, investigation of the 16S rRNA gene sequence in our study revealed that this gene's conserved region only had a few small nucleotide alterations. It is advised to carry out more research to find additional heavy metal resistance genes, such as the *czcA* and *ncc* genes in *E. coli* isolated from common carp in Sulaymaniyah province.

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Conflict of interest: The authors declare no conflicts of interest.

Authors contribution: ZKH designed study and the article's first draft was written by CHO and EDA. CHO performed experiments in the lab. The completed article was read and approved by ZKH and EDA.

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