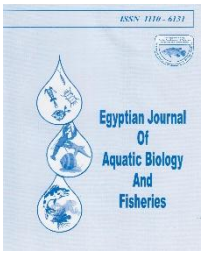
Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 27(4): 1047 – 1062 (2023)
www.ejabf.journals.ekb.eg



Molecular identification of some zoonotic bacteria isolated from fishes *Cyprinus carpio* L. and *Oreochromis niloticus* (L.)

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ARTICLE INFO

Article History:
Received: July 10, 2023
Accepted: Aug. 3, 2023
Online: Aug. 21, 2023

Keywords:
Zoonotic bacteria,
Aeromonas,
16S rRNA gene,
Oreochromis niloticus

ABSTRACT

Zoonotic bacteria isolated from fish cause many diseases, some of which are transmitted to humans, and it is a danger to public health in light of the recent spread of epidemics. The current study was designed to isolate and indentation bacterial species originating from two types of wild fishes (healthy and infected *Cyprinus carpio* and *Oreochromis niloticus*) in two different locations (Al-Mas`hab and Garmat Ali River) of water in Basrah, southern Iraq, during (2022) in two seasons of the year. The most prevalent species were identified in the two seasons (winter, and summer), the bacteria were isolated from the internal organs of fish (liver, kidneys, and spleen). The morphological and biochemical tests were used to identify the bacteria. The most prevalent species in the two types of fishes were indentation by targeting a specific 16S rRNA gene of the bacteria. The bacterial species successfully identified in *C. carpio* were *Aeromonas hydrophila*, *A. salmonicida*, *Vibrio parahaemolyticus*, and *Klebsiella oxytoca*. Five bacterial species in fish *O. niloticus* were detected (*A. bivalvium*, *V. parahaemolyticus*, *Pseudomonas* sp., *Citrobacter* sp. and *Shewanella algae*). The study showed that the increase in environmental pollution factors the pathogenicity of the disease has been shown to vary seasonally in the internal organs of healthy and infected fish.

INTRODUCTION

Bacteria of zoonotic cause diseases transmitted to humans (Han *et al.*, 2016). The problem of environmental pollution remains the main factor in the increase of zoonotic diseases, both bacterial, parasitic, and viral (Wolfe *et al*., 2007; Raissy .2017). Opportunistic pathogenic bacteria (Aeromonadaceae, Vibrionaceae, Pseudomondaceae, Enterobacteriaceae) constitute the largest part of them and the risk of their increase threatens public health and facilitates stress and environmental pollution the increase of negative bacterial species that are resistant to antibiotics (Gauthier 2015, Regev *et al*., 2020).

Fish have considered one of the most important sources of protein providing about 16% of the animal protein consumed by the world (Al-Hajimi, 2021) as many people around the world depend on fish as a primary source of animal protein. The common carp (*C. carpio*)

found in Iraqi waters in abundance, in addition to the Nile tilapia fish (*O. niloticus*), has now spread in the south of Iraq (Mohamed. 2020).

Cyprinidae is one of the fish in Iraqi waters, and the most important family of commercial fish, collectively called Cyprinidae, it is the largest and most diverse family of fish in the world, with about 1,300 species of carp (Noga, 2010; Goncalves *et al*., 2019). Tilapia may be infected with various bacteria, including species of the genera *Vibrio, Aeromonas*, *Pseudomonas*, and *Streptococcus*; some genera may be present also in healthy fish (Meron *et al*., 2020).

Several bacteria genera have been found from the mucus coating the fish's exterior surface, including *Salmonella*, *Pseudomonas*, *Sarcina*, *Micrococcus*, *Vibrio*, *Serratia*, and *Aeromonas*. In addition, a substantial majority of these microbes have been reportedly isolated from the fish digestive tract, such as species of the genera *Vibrio, Pseudomonas, Salmonella, Escherichia, Staphylococcus, Streptococcus,* and *Vibrio* (Ramadan *et al*., 2018; Al-Shammari, 2017). Widely acknowledged that bacteria are frequently found inside fish, including internal organs like the kidney, liver, and spleen, as well as on fish surfaces like the skin and gills.

Zoonotic bacterial causes of diseases including ulcer syndrome leading to high mortality can also be a problem for human consumers and high economic losses (Shayo *et al*., 2012; Hanna *et al*., 2014). However, epidemics linked to bacterial infections, biotoxins, histamine, viruses, or parasites have implicated fish and fish items that were shockingly raw or undercooked.

Fish and similar products provide a potential health danger from a microbiological standpoint because they contain vital human pathogenic germs on or inside of them. The intake of inadequately prepared fish and incorrect handling could result in bacterial illnesses. In addition to human infections, bacteria are thought to be the main reason why fish deteriorates.

PCR is a technique for the rapid production of amplification of many copies of specific DNA. It is important to confirm the diagnosis, find appropriate treatments, and know the pathogenesis of the disease and zoonotic bacterial diseases (Farzadnia and Naeemipour, 2020). Due to physiologic abnormalities, fish typically succumb to opportunistic bacterial infection, and stress factors like inadequate nutrition, bad water quality, and overfishing can lead to fish diseases; zoonotic bacterial come in two varieties: native and nonnative (WHO.2012).

Therefore, it is necessary to conduct a bacterial survey to find out the extent of the spread of bacterial species of zoonotic origin to investigate the potential risks to public health caused by environmental pollution in Al-Mas`hab and Garmat Ali River for the presence of industrial waste and agricultural waste. Therefore, the main objective of the current study was to isolate and identify zoonotic bacterial associated with fish species from the immune systems in fish; the pathological species were diagnosed and detected by molecular detection (by PCR method) of pathology bacteria most prevalent isolates and an evolutionary tree was made for each species separately, which was isolated from two types of fish.

MATERIALS AND METHODS

Fish samples

Healthy and infected fish belonging to two species of fish were collected in two different sites Al-Mashab and Garmat Ali rivers in Basrah. The fish species include common carp, *C. carpio* (total weight = 41.0 ± 9.5 g), and Nile tilapia, *Oreochromis niloticus* with total weight = 31.0 ± 5.0 g. (Table. 1) were randomly collected, December-July 2022 for

winter(December ,Jan, Feb) and summer (May, Jun, Jul). Clinical signs of disease were recorded and fish were transported to the laboratory of the Fisheries and Marine Resources Department, Agriculture College, University Basrah for microbiological analyses.

Environmental Parameters

Samples of water were collected using sanitized, glass bottles in two different sites (Al-Mas`hab and Garmat Ali rivers) in Basrah. The physicochemical properties of the water were assessed immediately after the lab. The Winkler method was used to gauge the amount of dissolved oxygen (DO). The YSI-665MPS was used to test the water's pH, salinity, and temperature. A technique suggested by the organization service for Public Health in America was used to assess biological oxygen demand (BOD5) (APHA. 2005).

Isolation of bacteria

Bacteria samples were isolated from the internal organs, liver, kidney, and, spleen of diseased fish, which were aseptically, and healthy fish by taking 1.0 g of tissue. The tissues were mashed using a manual homogenizer and placed in sterile test tubes containing 9 ml of sterilized distilled water. The test tubes were shaken using a vortex device for 2 minutes, then left to settle for 5-10 minutes, and then planted on culture media by spreading method. Tryptic soy agar (TSA), thiosulfate-citrate-bile salt-sucrose agar (TCBS), nutrient agar (NA), and Mac Conkey agar then the media were incubated in an incubator at a temperature of 37°C for 24 to 48 hours for bacteria.

Identification of bacteria

Microscopic properties and biochemical tests

Gram stain was used to examine the isolated bacteria for studying the macroscopic properties (Ashikuzzaman *et al*., 2015). The biochemical tests (oxidase, catalase, Voges Proskauer, catalase, and in-dole tests) were performed according to AbdeIslam *et al*. (2023). Identification of bacteria by VITEK@2 GN-ID System.

Molecular detection of the most prevalent pathological bacteria.

PCR Assay:

The DNA purification kit is designed for the isolation of DNA from bacteria and uses universal primers (27F, 5′-AGAGTTTGATCCTGGCTCAG-3′) and (1492R, 5′-GGTTACCTTGTTACGACTT3-′) according to (Dong *et al*., 2017). The amplified PCR products and the ladder marker are resolved by electrophoresis on 1.2% agarose gel with 1.0 µl (Fig. 2) fluorescent stain for dsDNA (Promega) using the primers described above. Sequence analyses were performed using Sequencher (Korean company Macrogen) (Cheng *et al*., 2023). Gene sequences were identified using the database queries that were used to estimate the degree of similarity with the sequence of other genes by the NCBI website (http//: www.ncbi.nem.nih.gov). Phylogenetic trees and sequence analyses were constructed by the neighbor-joining method using the software MEGA (Macrogen).

RESULTS

Water quality

The study was conducted During the period from December 2022 to July 2022 in which there is a fluctuation in water quality parameters in the aquaculture throughout the sampling sites (Table. 2). The fish in sites Al-Mas`hab and Garmat Ali River (Fig.1). Species of fish the number of caught, total fish and infected fish during (winter, and summer), and the percentage of infection (Table. 1). during a stressful event which causes the fish to become

susceptible to disease infection, always exposed to a variety of stressors which including high stocking density, handling, transportation and on the other hand, fish immunity is reduced.

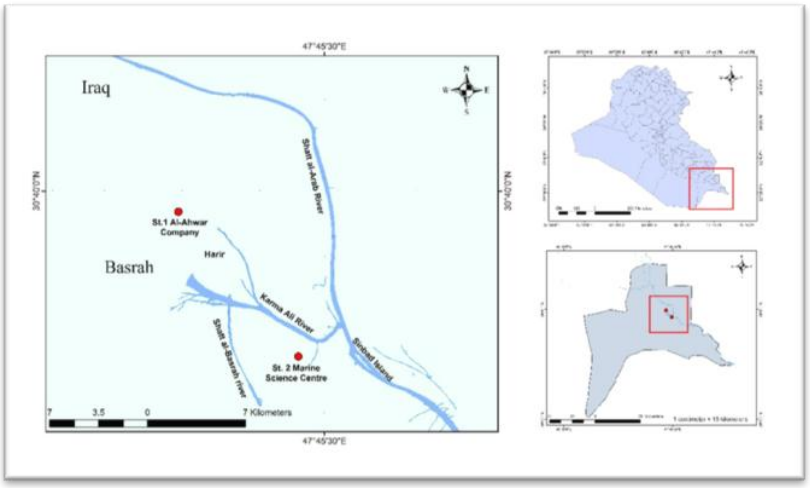

Fig. 1. Map of the study sites.

Table 1. Species of fish the number of caught, total fish, and infected fish during (winter, and summer), and the percentage of infection.

C. carpio	Season	Total fish	infected	percentage of infection %
	Winter	198	38	23.29
	Summer	130	25	12.62
O. niloticus	Winter	90	20	22.22
	Summer	113	12	10.61

Table 2. The water parameters of the studied stations

	Garmat Ali River*						
Param.	Des	Jan	Feb	Apr	May	Jun	Jul
Temp.	15.9	16.2	18.4	22.2	24.31	28.9	28.02
Sal	3.31	3.5	4.33	4.9	5.92	6.4	6.11
pH	8.11	8.21	7.9	8.8	8.6	8.7	8.2
DO	8.8	8.91	8.11	8	7.89	6.82	6.12
BOD	2.33	3.41	3.11	4.32	4.44	5.13	5.01
NO_3	2.1	2.23	3.12	2.5	3.4	5.21	5.91
	Al-Mas`hab River*						
Param.	Des	Jan	Feb	Apr	May	Jun	Jul
Temp.	16.7	15.1	17.5	20.6	24.4	27.9	29.11
Sal.	2.9	3.1	4.3	4.9	5.5	5.7	5.9
pH	8.12	8.02	7.93	8.11	8	8.14	8.19
DO	8.12	8.5	7.92	7.5	7.7	6.34	6.33
BOD	3.02	3.28	3.21	3.42	4.12	5.1	5.09
NO_3	2.32	3.12	4.77	3.21	4.32	5.21	6.06

*no statistically significant differences ($P > 0.05$) in the environmental factors between the study stations, significant differences ($P > 0.05$) in the environmental factors between the months of the year (SPSS, two-way ANOVA).

Table 3 shows the biochemical tests for the gram-negative bacterial spp. (Vibrio spp., *Aeromonas hydrophila*, *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, and *Proteus* spp.) isolated from examined fishes.

Table 3. Results of biochemical tests of bacterial spp. isolated from fish.

Biochemical tests	*Vibrio* spp.	*A. hydrophila*	*E. aerogenes*	*K. oxytoca*	*E. coli*	*P. aeruginosa*	*C. freundii*	*Proteus* spp.
Gram- stain	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+		+	+
Oxidase	+	+	-	-	-		-	-
Voges Proskauer	-	-	-	-	-	-	-	V
Methyl Red	+	+	+	+	+	+	+	+
Indole	-	-	-	+	+	-	-	-
Motility	+	+	-	-	-	-	-	-

+ = Positive, - =Negative, V = Variable +/-

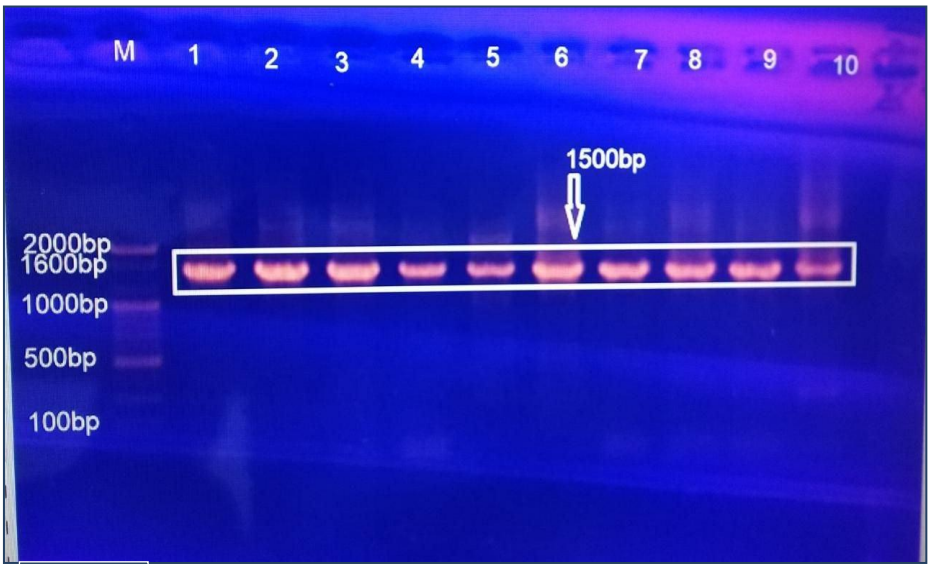

Fig. 2. Agarose (1.5%) gel electrophoresis analysis of the PCR products from 16S rRNA gene of bacteria with universal primers.
Lane (1: DNA marker (1500bp ladder)
Lane (2): (1, 2, 3, 4, 5, 6, 7, 8, 9, 10) Positive isolates of species bacteria.

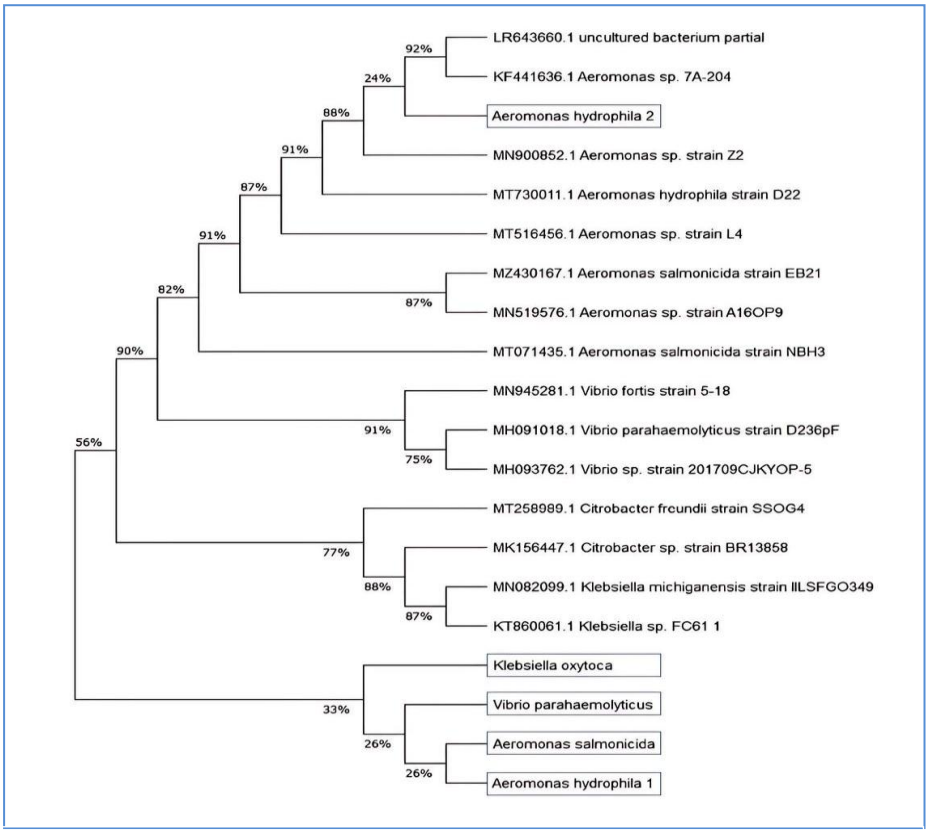

Fig. 3. Phylogenetic trees based on the partial 16S rRNA gene sequences of bacteria that caused infections and disease symptoms in fish. *C. carpio*

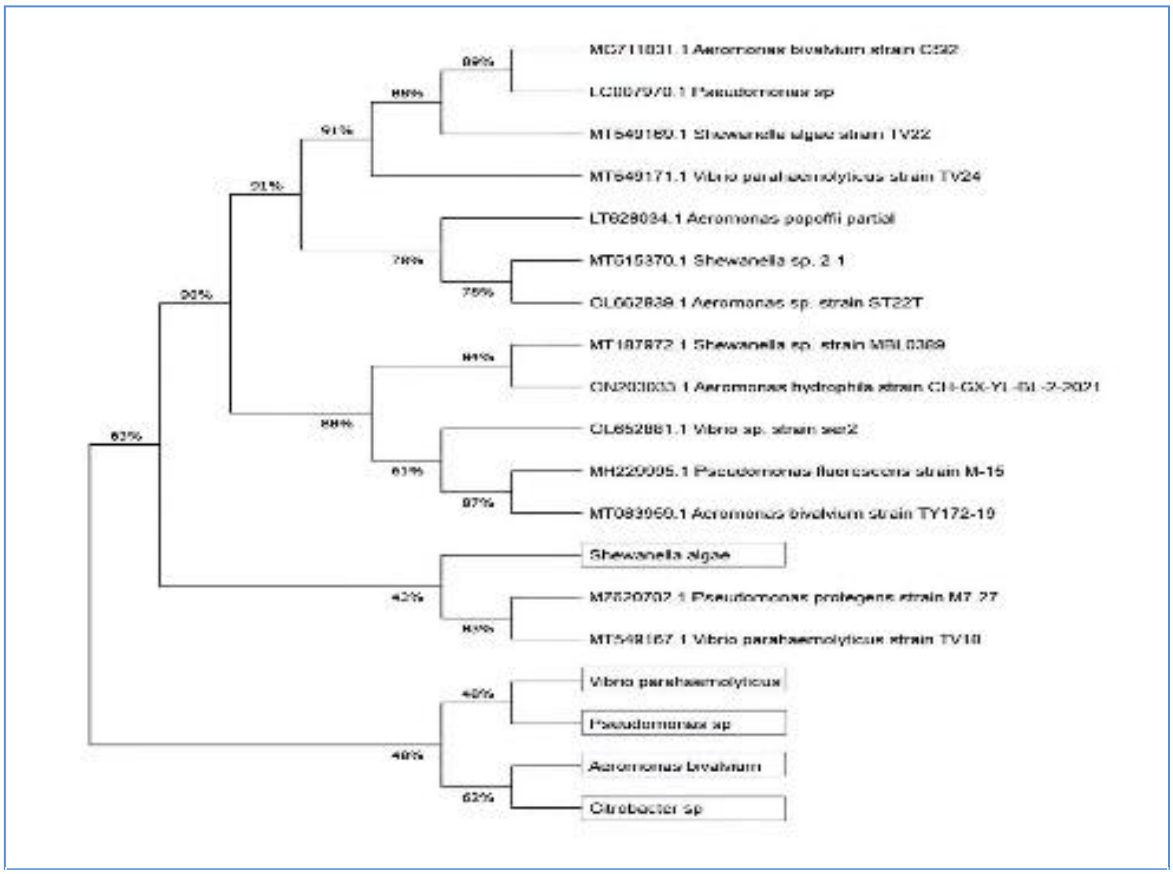

Fig 4.Phylogenetic trees based on the partial 16S rRNA gene sequences of bacteria that caused infections and disease symptoms in fish. *O. niloticus*.

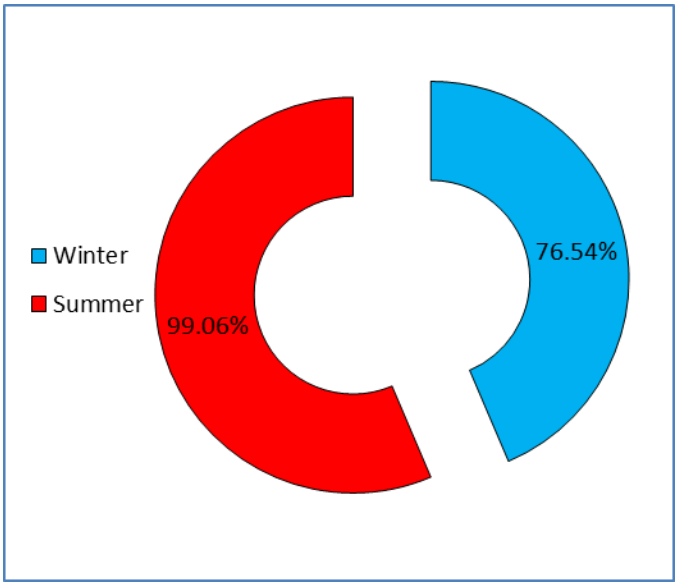

Fig. 5. The total percentage of the bacterial diversity found in healthy and infected fish belonging to two species of fish were collected in two different sites Al-Mas`hab and Garmat Ali River, fish species include *C. carpio* and *O. niloticus* for two seasons (winter and summer).

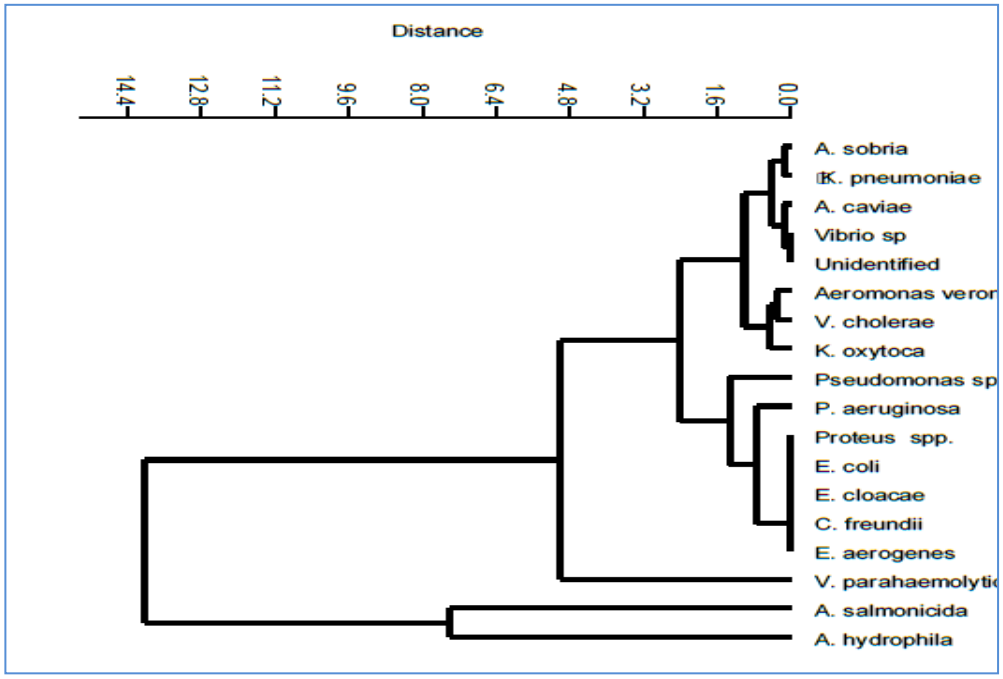

Fig. 6. The percentage of bacterial isolates that infected fish *C. carpio* with clinical signs in the skin and internal organs.

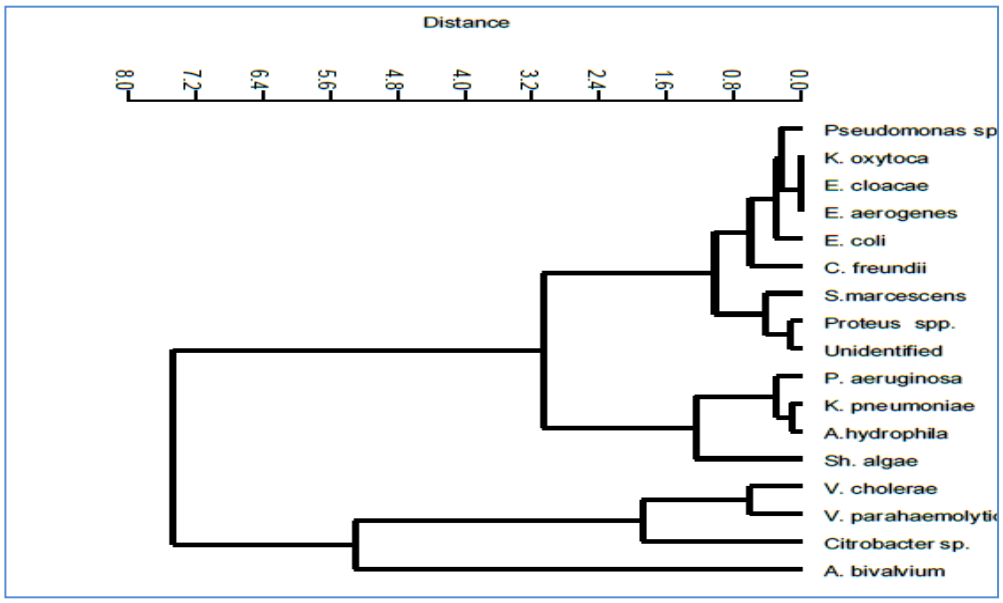

Fig. 7. The percentage of each bacterial isolate found in fish *O. niloticus* with clinical signs in the skin and internal organs

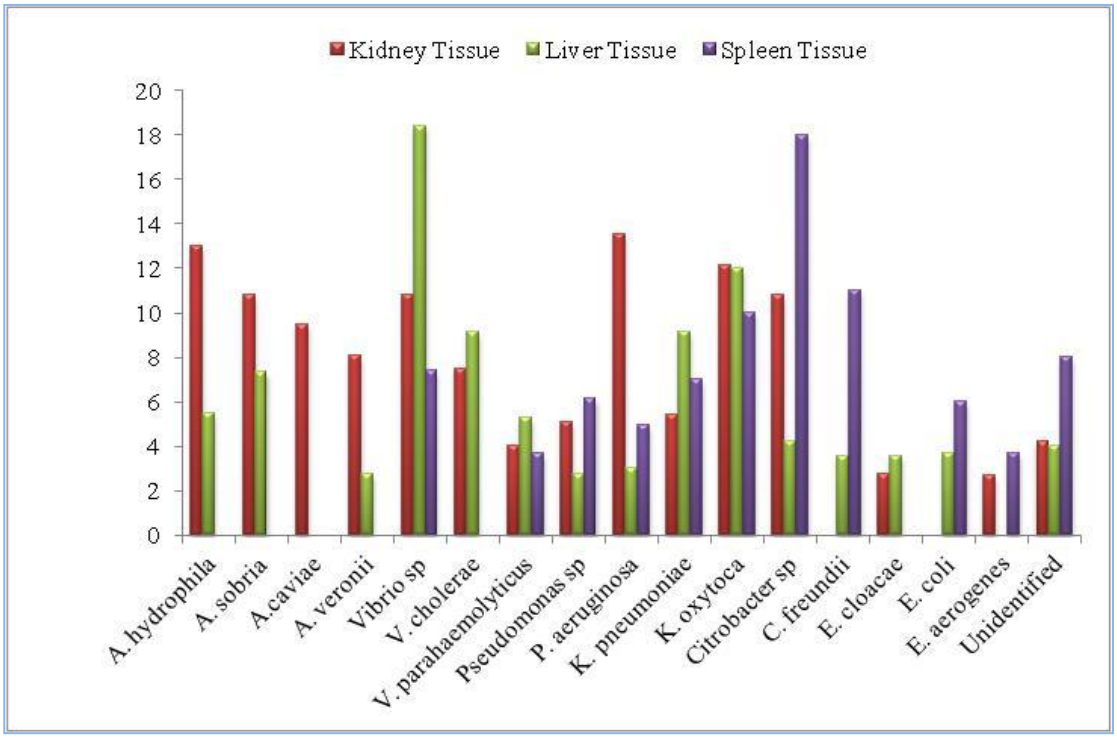

Fig. 8. The percentage of the number of bacterial isolates from internal organs (kidney, liver, and spleen) of healthy fish *C. carpio*.

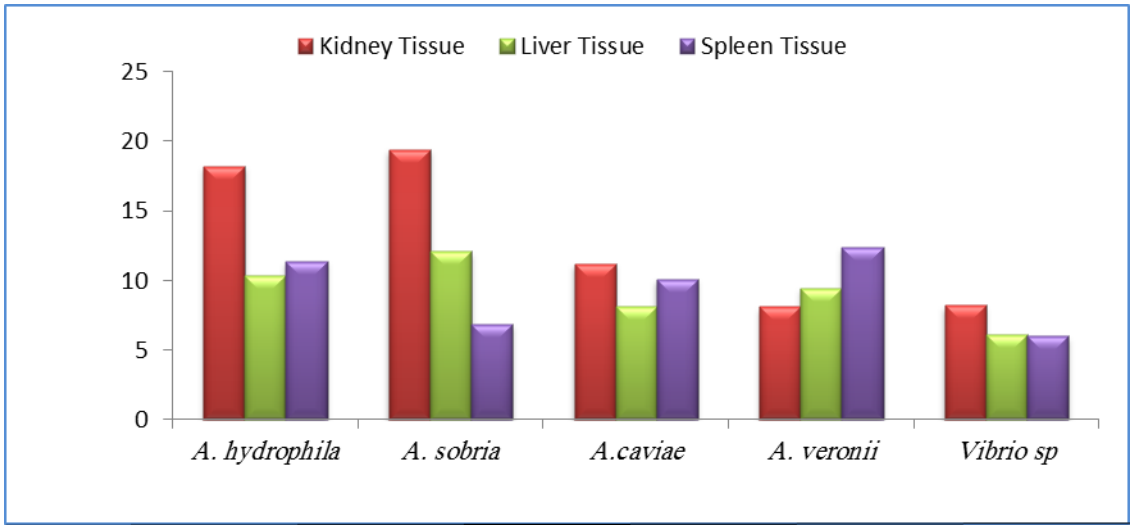

Fig. 9. The percentage of the number of bacterial isolates from internal organs (kidney, liver, and spleen) of healthy fish *C. carpio*.

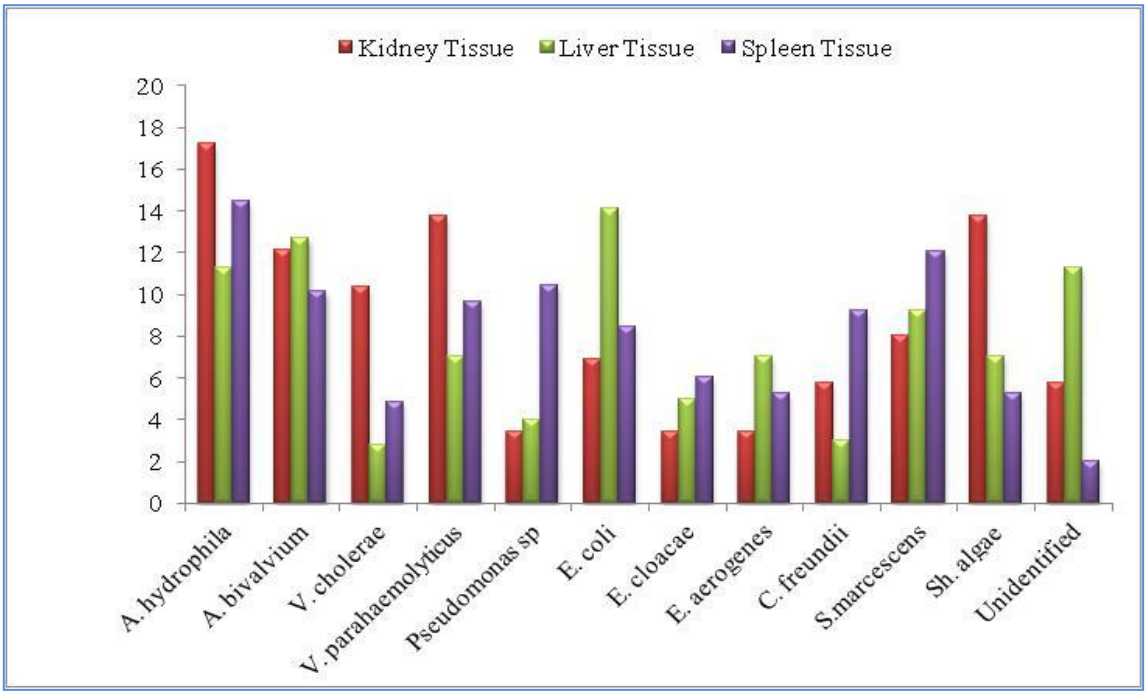

Fig. 10. Percentage of bacteria isolated from organs of healthy *O. niloticus* fish, which were isolated from the internal organs (kidneys, liver, and spleen).

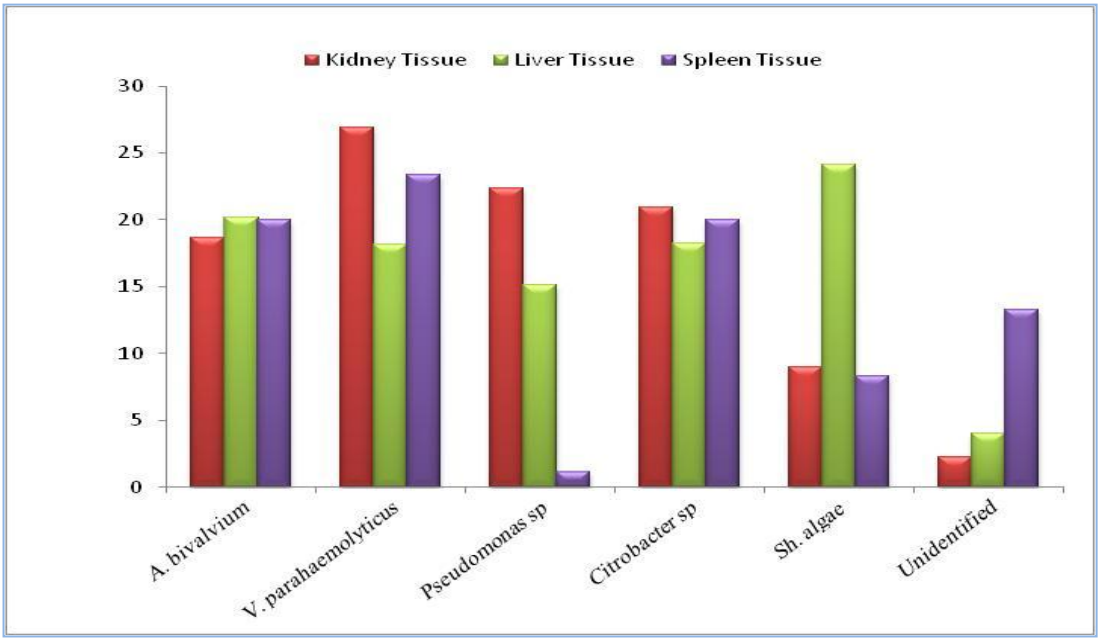

Fig. 11. The percentage of bacteria isolated from the organs of fish *O. niloticus.*

Clinical examination

The clinical examination of the collected infected *C. carpio* (Fig. 12) and *O. niloticus* (Fig. 13) showed body depigmentation, corneal opacity, frayed fins and tail, exophthalmia, detachment of scales, body ulceration, and hemorrhages all over the fish body, especially at fins and tails. Ulceration in the skin and hemorrhage, hemorrhage in the internal organs in some cases.

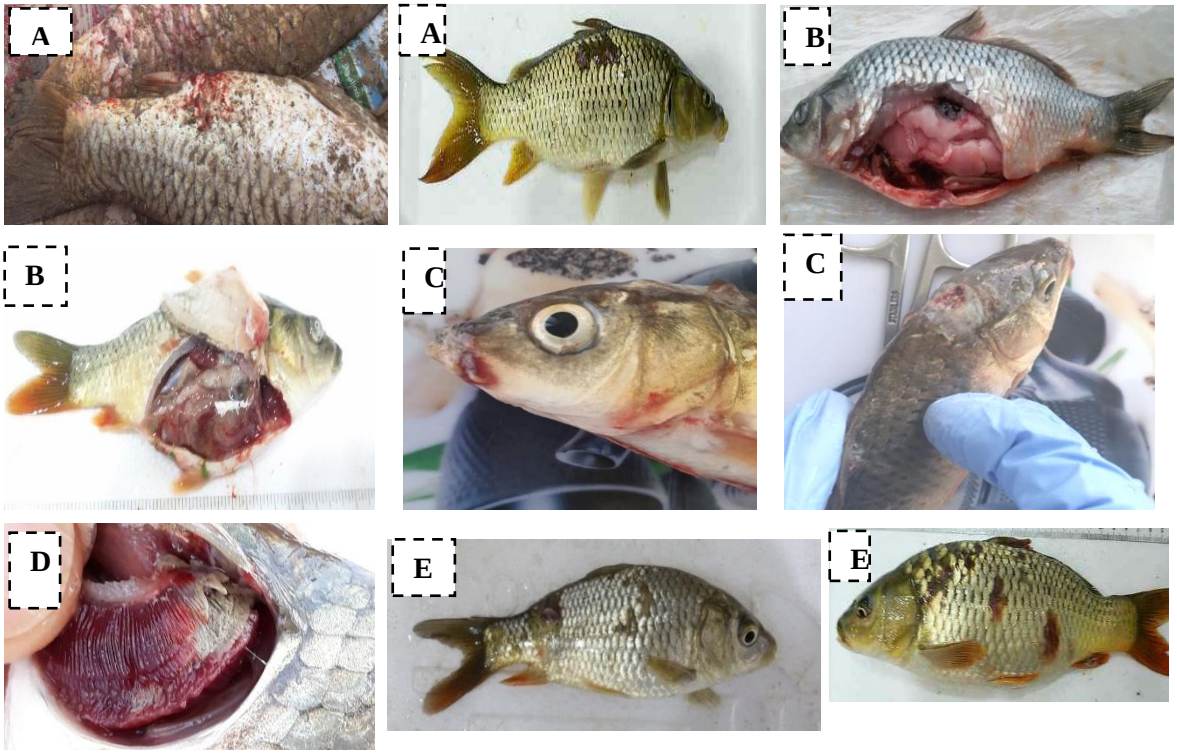

Fig. 12. Displays clinical signs of pathogens bacteria in *C. carpio*.

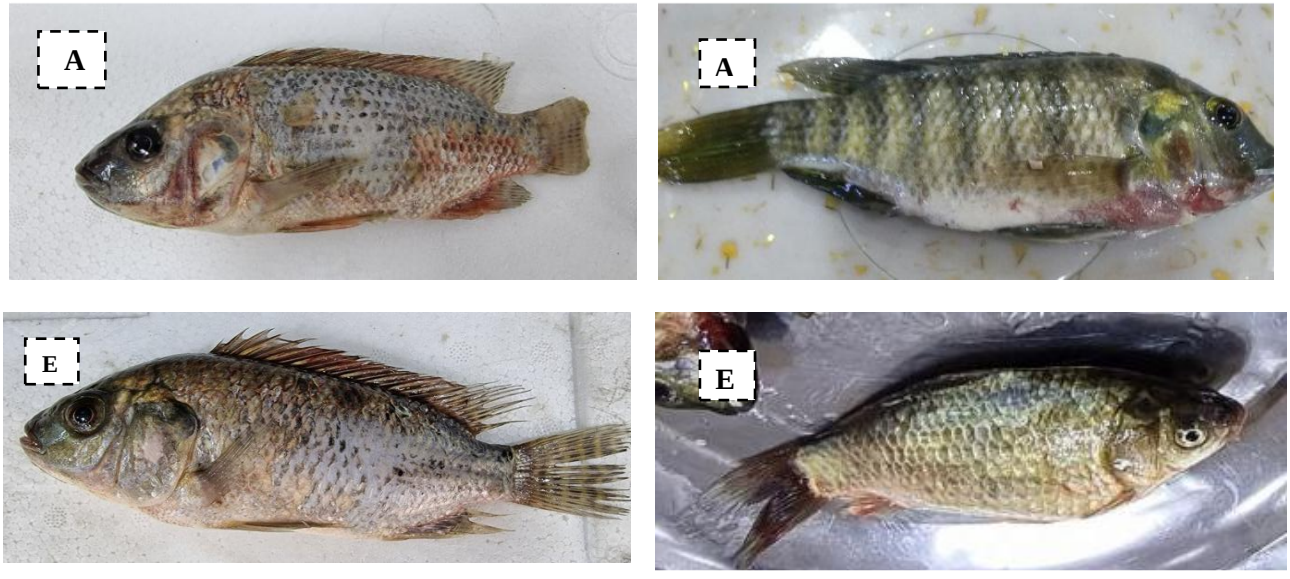

Fig. 13. Displays clinical signs of pathogens bacteria in *O. niloticus*.

Depicts clinical signs of pathogens bacteria in fish species, several symptoms were observed. (A) Ulceration in the skin and hemorrhage, (B) hemorrhage in the internal organs, (C) ulceration and skin erosion, (D) bacterial gill disease, and (E) Falling fish scales.

DISCUSSION

In this study, the presence of many important aquatic bacteria were investigated in two wild fish during a continuous survey, most of the positive samples were detected in the summer period, which can explain the higher prevalence of *Aeromonas* sp., *Pseudomonas* sp., *Vibrio* sp. However, fish infected by these bacteria could be a source of zoonotic risk for human health and are known to cause infections in humans with different degrees of severity, especially in immunocompromised this is in agreement (Tortoli, 2014).

In fish, commonly associated with elevated temperature, especially in temperate climes, generally, *Aeromonas* and *Vibrio* species are detected in summer but less common in winter (Fig. 5). This shows the total percentage of the bacterial diversity found in healthy and infected fish belonging to two species of fish were collected in two different sites Al-Mas`hab and Garmat Ali River, fish species include *C. carpio* and *O. niloticus* for two seasons (winter and summer). *Vibrio* sp. *Aeromonas* sp., *Enterobacter aerogenes, Klebsiella oxytoca* and *Escherichia coli*,. can be isolated from different organs spleen and liver, it has been suggested that these pathogens have an anonymity for, or are better detected, in kidney tissue.

This is in agreement with our results, where the prevalence of both pathogens in the kidney was higher than in liver tissues in most of the examined fish species. the percentage of the number of bacterial isolates from the internal organs kidney, liver, and spleen of healthy fish *C. carpio*.(Fig.8) the numbers of bacteria *Vibrio sp*. in the liver tissue were more prevalent; but in the spleen tissue, the *Enterobacteriaceae* bacterial species were more than the rest, which was similar in tissue, and the percentage of bacteria isolated from organs of healthy *O. niloticus* fish, which were isolated from the internal organs (Fig. 10).

Further isolation and characterization of bacterial species (Table. 3**)** is needed from this fish to understand its genetic properties necessary, phylogenetic analyses, based on the 16S rRNA gene sequences, revealed that all detected identical sequences in 16S rRNA gene 99.8% identity, (Fig . 3)Phylogenetic trees based on the partial 16S rRNA gene sequences of bacteria that caused infections and disease symptoms in *C. carpio*, and Phylogenetic trees based on the partial 16S rRNA gene sequences of bacteria that caused infections and disease symptoms in *O. niloticus* (Fig.4) The phylogenetic analysis of bacterial reveals a clear separation between the wild species *C. carpio*, and *O. niloticus*
By using (PCR) amplification and direct sequencing of 16S rRNA products, Knibb et al. (Turenne *et al*.,2001) identified bacteria directly from infected fish.
This allowed both proper taxonomic assignment and has opened the way to molecular epidemiologic analysis at the same time. However, even though this gene is still considered a key standard for bacterial identification (Gillman, *et al*.,2001) as more sequence information has accumulated over time, it has become evident that the resolution power of 16S rRNA sequences alone

The liver was pale in some fish, while in others it was hemorrhagic congestion. The spleen and kidneys were crowded and enlarged. in some fish. The intestines were hemorrhagic, with ulcers in the skin. This study revealed that septicemic bacterial infections with Gram-negative bacteria prevailed in the Gram-positive isolates with (*Aeromonas, Pseudomonas, and Vibrio*). Infected *C. carpio* and *O. aureus* with *A.*

hydrophila, a bacterium that damages the gills, liver, and kidney, resulting in histopathological changes in the infected organs (Al-Shammari *et al*., 2019; Al-Shammari 2017). This is similar to the injuries to these fish in our current study. *V. parahemolyticus* causes illnesses associated with fish (Austin and Austin, 2007).

The clinical signs of disease (anorexia, subcutaneous hemorrhages, lethargy, and ascites), microbiological analysis based on phenotypic and genetic characteristics partial sequencing of 16S rRNA genes of all clinical isolates, and molecular genetics investigations of their relationships with other strains are crucial for proper diagnosis in fish to know the pathological symptoms of each bacterial species (Choby *et al*., 2020; Wyres *et al*., 2020). Carps, catfishes, tilapia, brackish water shrimp, and freshwater prawns are the major aquatic animals in Asia.

Fish and aquatic animals are highly affected by water and air temperatures and are a major reason for the survival and growth of fish (De Silva and Soto, 2009); Table (2) showed a rise in the summer and a decrease in the winter, due to the nature of the climate of Iraq, which consistent with Al-Saad *et al*. (2010). Tropical fish such as carp do not tolerate seasonal fluctuating temperatures, especially in summer, lack of oxygen, as well as high salinity (Wyres *et al*., 2020). These environmental factors have a significant impact on the fish and cause stress for them, which is one of the causes of the onset of disease in fish.

In this study, the widespread distribution of negative bacteria in the study areas such *as A. hydrophila, A. Salmonidae, A. veroni; Vibrio, Escherichia, Salmonella,* and *Klebsiella*, make their spread to new hosts proportional to the geographical distribution (Odeyemi and Ahmad, 2017). These bacteria have caused many diseases in fish, such as vibriosis caused by *Vibrio* bacteria and hemorrhagic septicemia caused by *Aeromonas* bacteria (Gauthier, 2015).

Pathological bacteria, especially *Aeromonas* bacteria, can enter the circulatory and lymphatic system during infections and cause bleeding blood this leads to the exit of fluid from the circulatory system to the tissues and to maintain their balance, some organs such as the kidneys secrete cytokines during the inflammatory response (Austin and Austin 2016, Al-Shammari, 2021; Weir *et al*., 2012). Therefore, it is important to know people who suffer from special medical conditions, such as the ease of transmission of bacteria to them through contact, wounds, or ingestion of contaminated water. It is necessary to assess the risks of zoonotic bacteria to which fish are exposed and the genetic description is considered an accurate description to determine the disease and the features of the disease (Al-Gendy and Klena, 2008). This study is the first of its kind to investigate the distribution of zoonotic bacteria for two seasons in Basra and the causes of the spread of bacteria from the negative races, which are the cause of most diseases, a great threat to public health, as described (WHO, 2021).

CONCLUSION

The current study indicated that the distribution of zoonotic bacteria, especially negative species of them on a variety of hosts of fish in Basra governorate, has a danger to public health, which is the first study of its kind in Iraq showing their spread and the causes of their appearance, especially opportunistic ones, which helped the emergence of stress to which the fish were exposed.

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