Iraqi Journal of Veterinary Sciences

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Molecular and pathological study of *Providencia alcalifaciens* isolated from *Cyprinus carpio*

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Article information

Article history:
Received 27 March 2025
Accepted 08 August 2025
Published 17 September 2025

Keywords:
Phenotype
PCR
Providencia spp
Carp fish
Histopathological

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Abstract

A disease recorded in *Cyprinus carpio* during the survey period from September to December, 2024 at the commercial fish earthen pond in Khazir area in Erbil province -Iraq with high mortalities of 75 %. The goal of the study was to isolate and molecular identify the pathogen that causes fish mortality and study the histopathological alteration in the liver and kidney (naturally and experimentally infections). Gram-negative bacteria and urease-positive with *Providencia* bacteria colony as small, convex, pale in color, and non-fermenting lactose sugar was isolated from MacConkey agar medium. The diseased carp was lethargy with petechial hemorrhage at the basis's fins. The pathogen species was identified via biochemical responses and amplification 16S rRNA locus by PCR, a novel genome PV101576 and PV101577 were identified in (naturally and experimentally infections) respectively, and phylogenetic analysis reveal its relationship with *Providencia alcalifaciens in* NCBI database. Experimental infection assays with naturally infection isolate were conducted and pathogenicity by immersion 6×10^8 CFU/ml was demonstrated in healthy carp fish for seven day. Histopathological analysis reveals proliferative melanomacrophage, infiltration of inflammatory cell with renal and hepatopancreatic steatosis, renal cyst forming with circulatory disturbances. Conclusion of this study investigated that the Providencia-infection may be due to handling practices and ecosystem pollution, and from feces of the human and animal from adjacent fish culture. Therefore, Providencia isolate is regarded as an opportunistic bacterium for carp and lead to histological architecture alteration. This is the first investigation of Providencia that cause infections in carp farms.

DOI: 10.33899/ijvs.2025.158677.4204,

Introduction

According to reports from international organizations, the economic loss of fish production due to diseases may range from 15% to 40% in the event of an unintentional disease outbreak. In recent years, abnormalities in fish health have been considered a major problem to fish culture, primarily due to the development of numerous fish pathogens and their increase in resistance to antibiotics and chemical disinfectants (1,2). Bacterial diseases are among the most important infectious diseases that affect aquatic organisms, such as *Aeromonas spp*., *streptococcosis*, and *Vibriosis* (3-6), some of these bacteria cause high economic losses in aquaculture production (7). one of these bacteria is the *Providencia spp*. (8). *Providencia* belongs to the family Enterobacteriaceae and includes fourteen species (9). It is a gram-negative, motile, non-fermentative, urease-producing, opportunistic pathogen, isolated from feces of birds and poultry (10) as well as from various fish organs, as spleen, blood, gills, liver and kidney of Nile tilapia (*Oreochromis niloticus*) where the lesions of the disease is characterized by hemorrhages in the eye, fin and tail erosions, rot, dyspnea,

darkening of the skin color and reddening of the anal region, and lepidarthosis (11), liver and small intestine in cultured Lessepsian bloater (*Lagocephalus sceleratus*) (12), muscle and skin in rohu carp (*Labeo rohita*) (8). it has also been isolated from the shark *Triaenodon obesus* (13). *Providencia alcalifaciens* is known as one of the causes diarrheal infections in humans and cause diseases of animals as enteritis in puppies which are died with one to two days, chickens in addition to cows (14-15), Wang *et al*. (16) investigated that *Providencia alcalifaciens* cause hemorrhagic pneumonia in piglets.

Although the *P. alcalifaciens* is generally found in sewage, soil, and water but there is no reports about its ability to infected aquatic animals, so in recent months, The aims of our research group has been to isolate and molecularly identify the *Providencia alcalifaciens* as chargeable for disease in carps earthen culture and evaluation the pathogensisi and its impacts effects in carps internal organs.

Materials and methods

Ethical approval

The methodology and fish handling was approved by the Institutional Animal Care and the Committee was UM.VET.2024.091 University of Mosul College of Veterinary Medicine.

Fish sampling

The diseased common carp (*Cyprinus carpio*) average weight about 3 kg raised in earthen ponds were collected from Khazir area in Erbil province. The study started from September to December in 2024, fish showed petechial hemorrhagic at the base of the pectoral and pelvic fins with mortality rate reach to 75%. Fish kept in polyethylene bags with aerated water until reached to the microbiology laboratory in College of Veterinary Medicine -University of Mosul, Iraq.

Bacterial isolation and phenotype criteria

Fish were anesthetized by adding MS-222 (150 mg/ l), in the aquarium and then humanely sacrificed by cervical dislocation (17). Under aseptically technique, thirty samples were collected from internal organs (liver and kidney), 15 samples of each one organ. Following a 24-hour incubation period at 37°C in Soya Trypticase Broth (STB), swab samples were streaked in Blood Base Agar (BBA). Additionally, Gram stains and optical microscopy observations were conducted (18). Seeding was then conducted in selected culture media. Agar McConkey (Oxoid). Additionally, Gram (−) pathogens cultures, biochemical characterization was done using Vitek 2® (BioMérieux, Marcy-I'Étoile, France), this bacteriological tests were depended also in re-isolate (experimental infection) for confirmation the isolation and validation of pathogenicity. Bergey's guide of definitive bacteriology was used to identify the positive isolates (19), that are maintains on trypticase soy agar for confirmed bacterial isolate from both naturally and experimental infections by using PCR techniques.

Molecular analysis

DNA extracted from all confirmed *Providencia spp* bacterial isolates. The DNA extraction was done using AddPrep Genomic bacterial DNA Extraction (Korea) according to company instructions. The DNA concentration was determined using nanodrop (NanoPhotometer® N50/ Germany), and all DNA was stored in -80°C until used. To detect *Providencia spp.,* a 25 µl PCR mixture was utilized, which included 10 µl of Master Mix (Promega, USA),1 µl primers (for each forward and reverse), 8 µl of Danase-free water, with 5 µl of extracted DNA. All primer sequences were listed in table 1. The amplification programmed was done by using conventional Polymerase chain reaction PCR (sensoquest, Germany) as in table 2 (20). Agarose gel electrophoresis 2% (Applied Biosystems, USA) with 2µl ethidium bromide in TBE buffer was used to examine all PCR results. In order to see the DNA bands, a UV transilluminator was used. The data result of 16s RNA gene amplification were sequenced; to obtain the sample similarity with Center for Biotechnology Information (NCBI) database the Local Alignment Search Tool (BLAST) has been used for analyzed the data.

Table 1: Primers used in this study

Primer		Sequence (-) Product	Amplicon size	References
Psp16s	F	ACCGCATAATCTCTTAGG	515 bp	(20)
	R	CTACACATGGAATTCTAC		

Table 2: The PCR setting program of amplification

Type of PCR	Initial denaturation	Temperature/time (°C/min)			Final extension
		Cycle numbers 35			
		Denaturation	Annealing	Extension	
Psp16s	94/5	94/0.3	50 /0.3	72/0.3	72/5

Experimental infection of bacterial isolate

Immersion route of exposure were followed to investigate and evaluate the pathogenicity of isolate in healthy *C.carpio* according to the Ramkumar *et al*. (21) protocol. The bacterial count was determined by standard dilution and plating methods (22). Healthy fish (total number was 20) (23) were brought from hatchery in Ninevah government and transported with aerated water to fish laboratory in Veterinary Pathology and poultry diseases-College of Veterinary Medicine -University of Mosul-Iraq, maintain in aquarium for adaptation with dechlorinated continuous aerated water and feeding for one week (24), the fish were divided in two groups: control group in which fish kept in sterilized freshwater alone in infected group fish was exposed to 6×10^8 CFU/ ml for 7 day.

Histopathological analysis

To evaluation the impacts effects of bacterial isolate in tissue architecture (after sure there wasn't parasitic or fungal infections), a histopathological analysis of the metabolic and detoxification organs (liver and kidney) of naturally and experimental infections were performed. The collected fragments specimens, liver and kidney were preserved in 10% buffered formalin for 48 hours, after which they were treated with ethanol for dehydration, then immersed in xylene and embedded with paraffin wax for sectioning with a microtome at 5µ thickness, and then stained with routine stains (25).

Results

Laboratory tests were conducted on thirty fish samples, including 15 liver samples and 15 kidney samples, which suffered from a high mortality rate of 75%. *Providencia* bacteria were diagnosed among the ten bacterial isolates as shown in the following table (Table 3).

Phenotype and microscopical bacterial characterization

The naturally and experimentally infected carp fish showed general clinical signs as decrease of food constipation consumption and lethargy with petechial hemorrhage at the base of fins, post mortem lesions revealed congested of the posterior kidney and liver paleness (Figure 1). Morphological diagnosis of *Providencia* isolates when grown on MacConkey agar medium showed that the bacterial colonies were small, convex, pale in color, and non-fermenting lactose sugar (Figure 1). While the results of blood agar culture showed that the colonies appeared as regular, round, convex, non-clumped colonies, and had an odor similar to the odor of ripe fruits that accompanied the culture on blood agar medium (Figure 1), while the results of microscopic diagnosis showed that they were short, Gram-negative rods with a light red color (Figure 2). This evidential characteristic of diseased common carp and colony morphology indicated *Providencia* infection.

PCR investigation

The extracted DNA was amplified to identify *Providencia* spp. molecularly using universal primers. The amplification PCR products showed the target identified 515 bp DNA fragment (Figure 3), which indicates the *Providencia* spp. infection. Positive results were detected in each sample from both infection fish naturally and experimental.

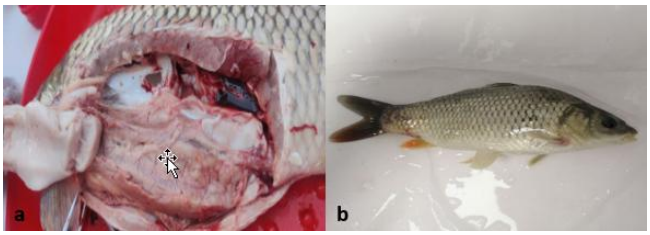

Figure 1: Pathology of *Providencia alcalifaciens* in *Cyprinus carpio* show petechial hemorrhage at the base of fins (a), with liver paleness and congested posterior kidney (b).

Figure 2: Phenotype features of *Providencia alcalifaciens* Colony morphology after incubation 24 hour on agars medium MacConkey and sheep's blood (a and b), (c) Negative Gram's stain of *Providencia alcalifaciens* from agar medium after overnight incubation.

Table 3: Number and percentage of bacterial isolates and their types from unhealth *Cyprinus carpio*

Isolate	Bacterial isolates	Number	Percentage%
Gram negative	*Providencia* spp	4	40
	Klebsiella pneumonia	2	20
Gram positive	*Enterococcus faecalis*	2	20
Mix	*Providencia* spp + *Klebsiella pneumonia*	2	20
Total		10	100%

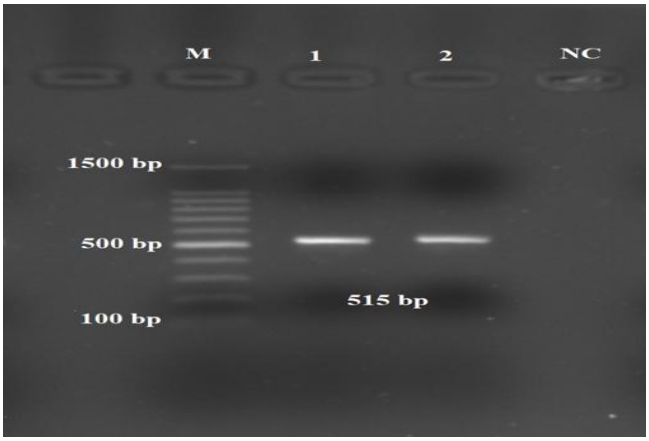

Figure 3: PCR product exhibit the amplification of 16S rRNA gene of *P. alcalifaciens* isolate on agarose gel (2% w/v) at 515 bp, Lane M -marker; NC: negative control, Lane1: natural isolated Providencia spp., Lane2: from immersion-infected fish (experimental).

The data of performing the Basic Local Alignment Search Tool (Blastn) progression in the (nr/nt) Nucleotide assembly database sited on the website www.ncbi.nlm.nih.gor.NCBI for specific sequencing analysis of recent local sequences (PV101576) and (PV101577) (for naturally and experimentally, respectively) of the *16S rRNA* gene for *Providencia alcalifaciens* that has a highly linked 99.81-100% identity to the pathogens isolated from different country and had sequencing with accession numbers in the GenBank (Table 4).

Following 1000 nucleotide sequence reconstruction using MEGA 11-Bootstrap analysis, the phylogenetic analysis of the 16S rRNA gene sequence of isolate Providencia that was entered into the NCBI GenBank under accession numbers PV101576 and PV101557 showed highly phylogenetic properties and a very close relationship 99.81-100% to other international sequences of the Providencia (Figure 4).

Figure 4: Phylogenetic tree of 16S rRNA of *Providencia alcalifaciens* using the Neighboard-Joining with the bootstrap values (%) are shown beside the clades, accession numbers are indicated beside the name of strains, and scale bars represent distance.

Table 4: Percentage distribution *of Providencia alcalifaciens* 16S rRNA *gene* on partial according to nblast in Genbank of NCBI

Sample Accession Number	Query Cover %	Identic Number %	Genbank Accession Number	Country
PV101576	100	100	PP858093.1	Nigeria
	100	100	JX827242.1	China
	100	99.81	CP151776.1	USA
	100	99..81	CP059348.1	China
PV101577	100	99.81	HQ407225.1	India
	100	99.81	MF399327.1	Iran
	100	99.81	MF399326.1	Iran
	100	99.81	HQ407280.1	India
	100	99.81	KJ396899.1	Portugal
	100	99.81	OR373609.1	South Korea
	100	99.81	AB682262.1	Japan

Histological analysis

Histopathology alteration of kidney of the *C.carpio* infected with *P. alcalifaciens* PV101576 and PV101577 reveal sever infiltration of leukocytes, and damage of renal tubules (coagulative necrosis), renal cyst forming), with hyperplasia of epithelial cells lining renal tubules as well as glomerular hypercellularity with increase Bowman's space, hemorrhage and renal steatosis, also there was investigated melano macrophage (Figure 5). While in the liver the histopathological lesions in both fish groups (naturally and experimental) infection characterized by sever vacuolar degeneration, distension of sinusoids and infiltration of inflammatory cells in hepatic pancreatic tissue with congestion pancreatic blood vessels in addition to hepatopancreatic steatosis (Figure 6).

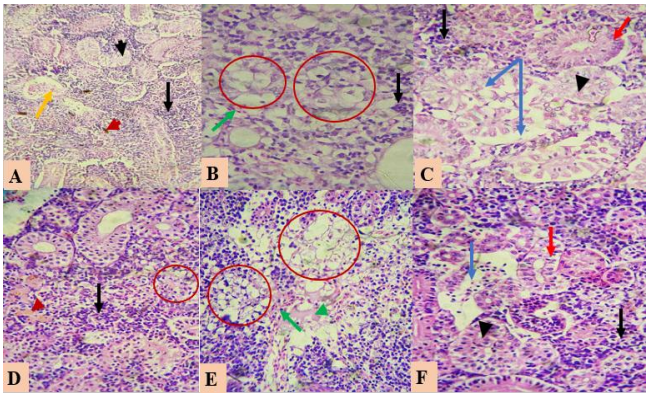

Figure 5: Histopathological examination of kidney in *C.carpio* infected with *P. alcalifaciens* PV101576 -naturally infection(A,100X-B), PV101577-experimental infection (D-F), reveal infiltration of leukocytes (black pointer), with congestion (yellow pointer), hemorrhage (green arrow), fibrinous serous exudate (green head pointer), coagulative necrosis (black head arrow), proliferative of melano-macrophage (red head pointer), beginning forming renal cyst (blue pointer), and hyperplasia of epithelial cells lining renal tubules (red pointer), 400X, H&E.

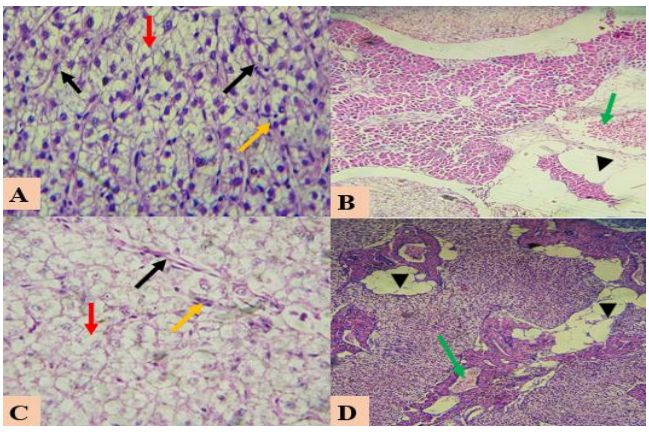

Figure 6: Histopathological examination of kidney in *C.carpio* infected with *P. alcalifaciens* PV101576 -naturally infection(A-B) 400X, PV101577-experimental infection (C 400X- D 100X), reveal infiltration of inflammatory cells (yellow pointer), sever vacuolar degeneration (red pointer) with dilatation of sinusoids (black pointer), congestion (green pointer) and steatosis (black head pointer), H&E.

Discussion

In current years, fishery industries and aquaculture have raped development and to produce large quantities of fish in an economically and biological well-organized approaches, but stressful culturing conditions as intensive density, management factors and environmental pollution make fish culture farms low resistance to different types of microorganisms cause infectious and outbreak diseases which often lead a highly economic loss (12,26). Many biological indicator responsible for identify the type of pollution (Heavy metals, or microbial), microbial indicator were represented by fungi, algae, worms eggs, and bacteria (27) as Enterococcus spp. and *Klebsiella pneumoniae* (28,29), so the percentage isolation of the current results (*Enterococcus faecalis* and *Klebsiella pneumoniae*) reveal that fish farms were polluted and may cause diseases to carp fish (30). Isolate of *P.alcalifaciens* at 10% from polluted environment (31). Bacterial septicemia is a main cause of disease and mortality in farmed freshwater fish, as Vibriosis, Streptococcal, and bacterial related to Enterobacteriaceae Aeromonasis, Pseudomonas's and Providencia (32).

In the recent study fish exhibit general gross lesions including petechial hemorrhage at the abdomen and base of fins (pelvic and pectoral), postmortem investigation revealed severe congested enteritis and accumulation ascites fluid, illness fish reveal yellow coloration liver and congested kidney (33,34), previous study reported that providencia cause septicemia in Salvadoran crocodiles (35) and species of turtles (36,37). Both bacterial invasion and colonization were responsible for the interior pathological lesions that developed on illness fish and distribution via hemolymph also, or may related to bacterial ability to produce cytolethal toxins (20,38,39).

Four from fifteen isolate were grown on MacConkey agar medium giving small convex, pale in color colonies and on sheep blood agar giving regular, round, convex colonies which are phynotype features of *Providencia alcalifaciens* as described by (40), and for confirmation PCR identification was performed. Phylogenetically, the family of Enterobacteriaceae has a highest similarity (16S rRNA gene sequence similarity), particularly with the members of genus Providencia >98.1% (41). The PCR amplification using *Providencia alcalifaciens* specific universal 16S rRNA recognized four *Providencia alcalifaciens* strain as the PCR assay results in the amplification of 515 bp band for *Providencia alcalifaciens* (21,20).

Providencia species primarily found in aquatic environments and are cause dieseas in bird and mammal diseases. It has been possible to isolate the *P. vermicola* and *P. rettgeri* from diseases fish, few study on bacterial diseases investigated that *Providencia* spp. as an one of the generally pathogen that causes hemorrhagic septicemia complicated in freshwater species (21,42,43). The histopathological results of current study in both naturally and experimental infections reveal deleterious effects of *P.alcalifaciens* in the kidney and hepatopancreatic tissues of infected carp, this result agreements with the results in *Labio rohita* (21) Nile tilapia (10), septicemic diseased turtle (37). Pointed out that Providencia has the ability to secrete a type of toxin (Cytolethal distending toxin CDT) that causes cell elongation and expansion, as well as cause disturbances of actin of cytoskeleton which lead to abnormal cell

permeability which lead abnormal cell respiration characterized by reduction in the Sodium/Potasium- ATPase and Na^{+}, K^{+} and Ca^{+2} unbalance, this pathway alteration lead to necrosis (20,44), adaptive and defense response occur as compensatory mechanisms hyper trophy, hyperplasia or inflammatory cells infiltration these may be the cause decline oxygen and gas and exchange lead to hypoxia which is the general pathological key of cells injury (45). Renal steatosis result from accumulation of fat and ectopic adipocytes and accumulation in the sinuses blood vessels and a combined with blood vessels disturbances (46).

Conclusion

A pathogenic strain PV101576 and PV101577 were a novel isolate and molecular identification of *Providencia alcalifaciens* that isolate from *C.carpio* farms in Iraqi culture with haemorrhagic septicaemia, that mean that fish was susceptible to this species of genus Providencia and may cause diseases and deleterious histopathological effects in an internal organs as other bacterial specific to infected fish and may lead to economic losses, further studies about the antibiotic sensitivity, virulence genes, and molecular pathway reports could supported a well understanding about the pathogenicity of *P. alcalifaciens* in fish culture.

Acknowledgements

The authors are thankful to Faculty of Veterinary medicine /University of Mosul, Mosul, Iraq.

Conflict of interest

There is no competing interest in this research paper.

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دراسة جزيئية ومرضية للبروفيدنسيا ألكاليفاسينس المعزولة من اسماك الكارب الاعتيادي

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الخلاصة

مرض تم تسجيله في اسماك الكارب الاعتيادي خلال فترة المسح من أيلول إلى كانون الأول ٢٠٢٤ في أحواض طينية للأسماك التجارية في منطقة الخازر في محافظة أربيل، العراق مع ارتفاع معدل النفوق بنسبة ٧٥٪. أجريت هذه الدراسة لعزل والكشف الجزيئي للعامل المسبب للنفوق في مزارع الأسماك ودراسة التأثيرات النسجية المرضية في الكبد والكلى (للإصابات الطبيعية والتجريبية). تم عزل الجرثومة سالبة الجرام وموجبة اليورياز ذات مستعمرة صغيرة ومحدبة وشاحبة اللون لجرثومة بروفيدنسيا ألكاليفاسينس وغير مخمرة لسكر اللاكتوز في الوسط الزرع الماكونكي. كان سمك الكارب المصاب خاملًا مع وجود نزف نقطي على قواعد الزعانف. من خلال التفاعل الكيميائي الحيوي وتضخيم سلسلة التفاعل المتبلمرة للمورثة 16S rRNA تم الكشف عن النوع الممرض وتحديد الجينوم الجديد PV101576 و PV101577 (الاصابة الطبيعية والتجريبية على التوالي) وكشف التحليل التطوري في قاعدة البيانات المركز الوطني لمعلومات التكنولوجيا الحيوية عن علاقته بروفيدنسيا ألكاليفاسينس، أجريت اختبارات الاصابة التجريبية باستخدام عزلة من إصابة طبيعية، وثبتت قدرتها على الإمراض عن طريق الغمر بمعدل ٦*١٠^٨ وحدة تشكيل مستعمرة/مل في أسماك الكارب السليمة لمدة سبعة أيام. كشف التحليل النسجي المرضي عن تكاثر الخلايا الميلانينية، وتسلل الخلايا الالتهابية مع استبدال النسيج الكلوي والكبدي البنكرياسي بالدهون، وتكوين كيس كلوي مع اضطرابات في الدورة الدموية. خلصت هذه الدراسة إلى أن عدوى بروفيدنسيا قد تكون ناجمة عن ممارسات التعامل وتلوث النظام البيئي، ومن براز الإنسان والحيوان من البرك القريبة وبالتالي، تُعتبر عزلة بروفيدنسيا بكتيريا انتهازية لأسماك الكارب وهذه هي أول دراسة للبروفيدنسيا التي يمكن أن تُسبب المرض في مزارع اسماك الكارب.