مجلة سوزوسا العلمية المحكمة
Sozusa Peer-Reviewed Scientific Journal
ISSN: 3078 – 2767
المجلد الثالث، العدد الاول، سبتمبر 2025م
DOI: https://doi.org/10.66358/sozusa.v3i1.001

Re-isolation, identification, pathogenicity and antibiotic sensitivity during experimental Infection of Nile Tilapia (*Oreochromis Niloticus*) With *Proteus Vulgaris Bacteria* under some stress factors

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الملخص:

أجريت هذه الدراسة لعزل وتحديد وتوصيف لبكتيريا *Proteus vulgaris* المعزولة من عدوى اصطناعيه لسمك البلطي النيلي السليمة ظاهريًا. تم استخدام ستين سمكة بمتوسط وزن 50 جم. لاختبار مسببات الأمراض، تم حقن مجموعتين داخل الصفاق بنفس الجرعات، أي 0.06 مل (1.5 × 10^9 وحدة تشكيل مستعمرة/مل سمكة). تم تأكيد الإصابة ببكتيريا *Proteus vulgaris* في المجموعة (أ) عند درجة حرارة ماء 27 م° وظروف بيئية مواتية مع معدل نفوق 40٪، بينما في المجموعة (ب)، كما تم تأكيد الإصابة ببكتيريا *Proteus vulgaris* عند درجة حرارة ماء 37م °وظروف بيئية غير مواتية مع معدل نفوق 80٪. يشير هذا إلى تأثير الظروف البيئية غير المناسبة على صحة الأسماك والانتشار السريع للبكتيريا داخل جسم السمكة، مما يتسبب في الوفيات. تم أيضا إعادة عزل *Proteus vulgaris* المحقون من الكبد والكلى والأمعاء للأسماك المصابة وأجراء الاختبارات الكيميائية واختبار الحساسية للمضادات الحيوية.

الكلمات المفتاحية: سمك البلطي، بروتيس فولقارس، الظروف البيئية.

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Abstract:

This study was conducted to isolate, identify, and characterize the pathogenicity of Proteus vulgaris isolated from an experimental infection of Nile tilapia. Sixty Nile tilapia (*Oreochromis niloticus*) fish with an average weight of 50 g were used. To test pathogenicity, two groups were injected intraperitoneally with the same dose 0.06 ml (1.5×10^9 CFU/ml / fish). Pathogenicity was confirmed in group A of Proteus vulgaris at a water temperature of 27 °C and favorable environmental conditions with a 40% mortality rate, while in group B, pathogenicity was confirmed at a water temperature of 37 °C and adverse environmental conditions with an 80% mortality rate, this indicates the impact of unsuitable environmental conditions on fish health and the rapid spread of bacteria inside the fish's body, causing deaths. The injected Proteus vulgaris was re-isolated from the liver, kidney, and intestine of the infected fish.

Keywords: *Nile tilapia*, *Proteus vulgaris*, environmental conditions, experimental infection

1. Introduction:

Bacterial diseases were considered the main cause of high mortalities and economic losses among fish farms **(B Austin & Austin, 2007) ,** *Enterobacteriaceae* is a large family of some 25 genera and more than 100 species of facultatively anaerobic and gram- negative rods. The most important members of the family *Enterobacteriaceae* that are pathogenic to fish are *Yersinia ruckeri*, and *Edwardsiella sp*. However, recent reports showed that proteus has been implicated as potential fish pathogens. Proteus species are small Gram-negative rods, catalase positive, oxidase negative and produce acid from glucose by both oxidative and fermentative metabolism **(Daly & Aoki, 2011).** *Proteus vulgaris* is considered mainly a human pathogen, it was reported to produce active infections in fish. Previous studies reported that *P. vulgaris* causes diseases in several freshwater fish including, ulceration on skin of akame **(MUROGA, 1979)** , septicemia in carps **(Ramaiah and Manohar, 1988)** and mortalities in sharptooth catfish and tilapia **(Okaeme, 1989).**

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Proteus vulgaris is Gram negative microorganisms causing general signs of septicemia with severe economic losses in aquacultures **(Elgohary, I Abd Elatief, G Fadel, E Eissa, & A Mahmoud, 2020)**. it was isolated from ulcers in the fresh water fish **(Mandal, Mandal, Pal, Halder, & Basu, 2002)** and the naturally infected fishes with Proteus vulgaris showed hemorrhages in buccal cavity, body surfaces and base of the fins, fin rot, protruded hemorrhagic anus, congested gills and internal organs together with focal hemorrhage on the surface of the liver **(GALAL SAAD EL-DEEN, 2013) (Aya, 2013).**

Low flow conditions fragment streams into isolated pools, reducing available habitat and increasing fish density and organic matter decomposition (e.g., autumn leaf fall), sharply reduces dissolved oxygen (DO), stressing fish populations **(Dowling & Wiley, 1986).**

In the present work, experimental infection was done to isolation and identification, and to know the pathogenicity of *proteus vulgaris.* and studying the antibiotic sensitivity for these bacteria , An experimental comparison was made between artificial bacterial infections of fish under favorable conditions in terms of water flow, temperature control, and dissolved oxygen availability, and another artificial infection with the same bacteria of fish placed under unfavorable environmental conditions in terms of stagnant water, high temperature, and lack of dissolved oxygen in the water, and the effect of both conditions on the infection rate and mortality.

2. MATERIALS AND METHODS:

1.2. Experimental fish and set up:

60 Apparently healthy juvenile Nile tilapia (50±5g) were collected from a private fresh fish farm at Umm al Rizam municipality. Fish were acclimated in laboratory condition for 2 weeks to ensure that fish were healthy and none infected **(Austin and Austin 2007).** Fish were fed on pelleted ration at rate of 3% of their body weight a twice daily, The infection experiments were conducted at the laboratory of the natural resources and environmental sciences, Derna university, Quba branch (figure1) , Duration of the experiment was 1 month , used four aquaria of

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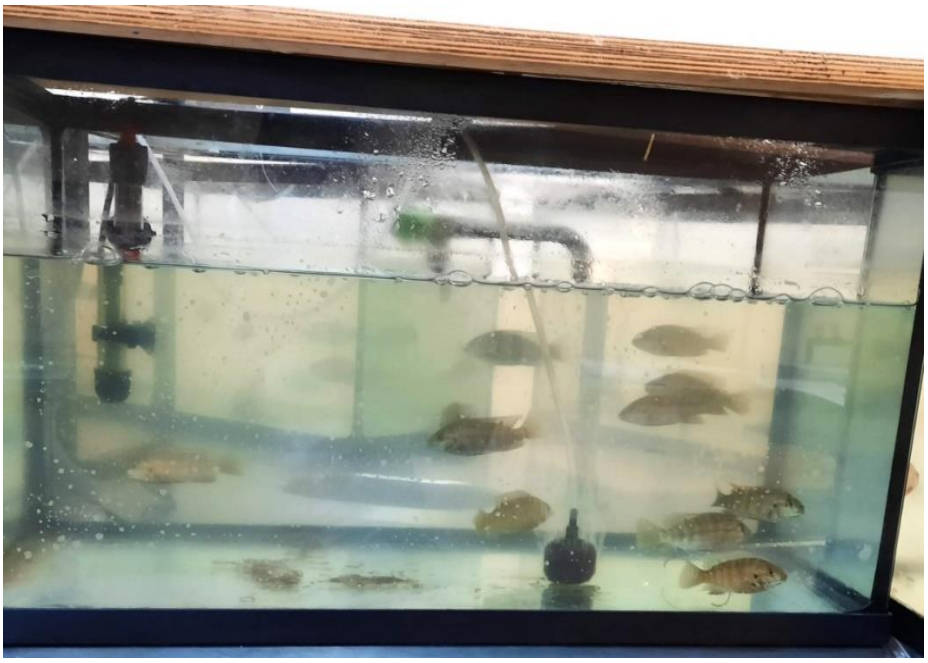
fiber glass each having 40 l capacity, the first aquaria (A) was supplied by electric pump and Water heater to set temperature at 27 C, Prior to the experiment the water was kept under circulation for 7 days. The second aquaria (B) put under conditions of stress such as high organic load, a water temperature of 37°C to To reduce levels of dissolved oxygen, and Water pump not installed for prevent stream flow and Scratches were made on the fish's body to facilitate bacterial infection, also Prior to the experiment the water was kept under circulation for 7 days, The third (C) and fourth (D) aquaria were provided with the same environmental conditions as the first basin.

Figure (1) : juvenile Nile tilapia for experimental infection



2.2. Bacterial strains:

The isolated Bacterial strains were identified previously as Proteus vulgaris were kept in BHI broth with 15% glycerol (El-Gomhurrhia Co, Cairo, Egypt) at -20ºC in the Central Laboratory in Alexandria, and it was brought to Libya. A Proteus vulgaris strain isolated from Nile tilapia, Strains was re-cultivated in BHI broth at 28°C for 12 hours to reach mid-log phase (optical density of 1.2 at 600nm wave length) to be diluted and used for experimental infection.

3.2. Bacterial count:

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Colony forming unit (CFU) counts of the bacterial suspensions were determined using spectrophotometery optical density (Thermo) (figure 2) at 600 values , tenfold serial dilution and plate count method (Elkamel & Thune, 2003).

Figure (2) : spectrophotometer

4.2. Experimental challenge to *O. niloticus*:

In a previous study, an intraperitoneal (I/P) injection of 1ml of bacterial suspension of $1X10^7$ cfu/ml, $1X10^8$ cfu/ml, or $1X10^9$ cfu/ml proved to be lethal within 7 days to all Nile tilapia. Thus, lower concentrations of the bacterial suspensions were used for experimental challenge. (GALAL SAAD EL-DEEN, 2013) (Aya , 2013).

Acclimated Nile tilapia were divided into four groups with 15 fish each group. The first group put in the first aquaria and was intraperitoneally injected with 0.06 ml of bacterial suspension of $1.5X10^9$ cfu/ml, while the second group was put in the second aquaria and I/P injected with 0.06 ml of $1.5X10^9$ cfu/ml and the third was I/P injected with 0.06 ml distalled water (Control Group) and the four group remained un-injected (baseline negative control). The behavioral and clinical abnormalities were recorded daily, for 15 days. The visible morphological changes of the internal organs were also recorded after autopsy of the moribund fish. Percent mortality was calculated after the fish kill. To confirm if the disease was caused by the injected pathogen only, re-isolation of the injected pathogens from the internal organs was carried out by sacrificing moribund fish.

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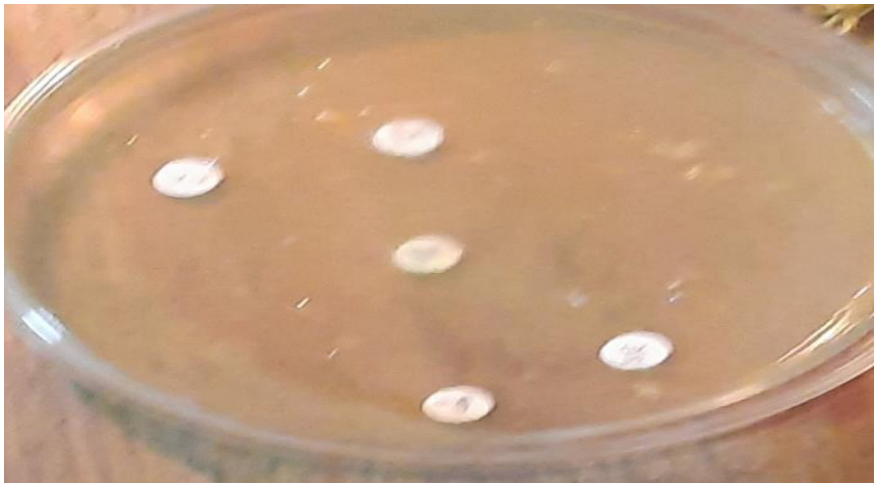
5.2. Bacteriological examination:

Loopfuls aseptically taken from liver, kidney, spleen and brain according to (Brian Austin & Austin, 2016) directly streaked on an xylose-lysine-deoxycholate (XLD) (Oxoid, Ltd), and brain heart infusion agar (BHI) (Biolife) for cultural, morphological, and biochemical characters, then followed by incubation at 25°C/ 24 hrs , pure bacterial of the isolates were identified by biochemical characterization following the criteria described in Bergey's Manual of Determinative Bacteriology (Holt et al., 1993) and performed by commercial API*20E kits (Bio-Merieux,France) following the criteria were described by (Elmer, Stephen, William, Paul, & Washington, 1997).

6.2. Antibiotic sensitivity test:

Sensitivity was determined by the agar diffusion method (Quinn, Markey, Carter, Donnelly, & Leonard, 2002) Quinn, et al., (2002) using 6 mm diameter commercial discs (Oxoid) included the following antibiotics' discs. The antibiotic discs were chloramphenicol, Gentamicin, Nalidixic acid, ampicillin, nitrofurantoin, Antibiotic sensitivity was tested on Mueller-Hinton agar with or without 3%NaCl (figure 3). Inhibition zones diameters were interpreted as sensitive, intermediate and resistant according to CLSI (2010).

Figure (3) : The antibiotic discs



3. Results:

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1.3. Fish:

1.1.3. The clinical signs :-

Sex dead Nile tilapia infected with P. vulgaris in the first aquaria (A), While the number of deaths was Twelve in the second aquaria (B), the fish showed irregular bleeding in the ventral part of the abdomen. In group (B) 8 fish examined, detachment of scales, rejection of the pectoral fin, bleeding on the body surface, and congestion of the gills were observed, while in (A) 5 fish examined the spleen was congested and enlarged. The liver was pale or greenish in 2 cases in (A) and congested in 5 cases in (B). The intestines of 3 fish were filled with serous-bloody fluid in group (A) while in group (B) was 7. The mortality rate reached 40 % (6) fish in group (A) while in group (B) was 80% (12) by the end of the experiment. The bacteria were isolated in pure culture from freshly killed and dead fish. There were no deaths or clinical signs of infection in both three and four control groups.

Table 1: Mortality and Bacterial Isolation

Parameter	Group A (Aquaria A)	Group B(Aquaria B)	Group C&D (Aquaria C&D)
Total Fish	**15**	**15**	**15**
Dead Fish	**6**	**12**	**0**
Mortality Rate	40% (6/15)	80% (12/15)	0% (0/15 each)
P. vulgaris Isolation	Confirmed (dead fish)	Confirmed (dead fish)	Absent

Group B's "bad" environment may have accelerated bacterial growth (increase virulance) (e.g., warmer water, abundant organic matter, low D.

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Group A's good conditions might limit bacterial virulence and slowing infection progression

Table 2: Clinical Signs Comparison

Clinical Sign	Group A (5 Fish Examined)	Group B (8 Fish Examined)
Ventral bleeding	Not observed	Observed (all fish)
Scale detachment	Not observed	Observed (all fish)
Pectoral fin rejection	Not observed	Observed (all fish)
Body surface bleeding	Not observed	Observed (all fish)
Gill congestion	Not observed	Observed (all fish)

Table 3: Summary of Organ Affections

Organ	Group A (Findings)	Group B (Findings)
Spleen	Congested/enlarged (5/5)	Not reported
Liver	Pale/greenish (2/5)	Congested (5/5)
Intestines	Serous-bloody fluid (3/5)	Serous-bloody fluid (7/8)

1. **Group B (Isolated)** showed **higher mortality (80%)** and **severe external/systemic signs** (bleeding, fin rejection, gill congestion).
2. **Group A** had **moderate mortality (60%)** with **internal organ involvement** (spleen, liver, intestines).

Control Groups (C & D) remained unaffected, confirming *P. vulgaris* as the causative agent

2.1.3. Bacteriological examination:

Bacteriological examination resulted in recovery of 15 isolates which were suspected to be P. vulgaris based on morphological and biochemical characteristics. Primary isolates grew well on BHI agar giving thin, colorless, transparent highly swarming colonies, P. vulgaris isolates produced yellow colonies in XLD media, all isolates were Gram-negative, motile, rods shape, The Phenotypic and Biochemical characterizations of all isolates of naturally infected O. niloticus are summarized in the following table.

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Table 4: results the biochemical test

Biochemical test	Result
Colony characters onto XLD medium	Yellow
Colony characters onto BH	colorless
ONPG B-galactosidase	Negative Colorless
ADH Arginine Hydrolase	Negative Yellow
CIT Citrate	Negative blue
H_2S H_2S production	Positive black deposit
IND Indole	Positive red
VP Vagous Prescour	Negative Colorless
GEL Gelatinase	Negative
GLU Glucose	Positive yellow
OX Cytochrome Oxidase	Negative
Gram stain	Negative rod shape
MAN Mannitol	Negative blue
INO Inositol	Negative blue
SOR Sorbito	Negative blue
RHA Rhaminose	Negative blue
ARA Arabinose	Negative blue
URE urease	within 6 hours positive

Table 5 :API E20 test result:

Triad																				
Tube	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	oxidse
Reaction	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	+	-	+	-	-
Point	**0**	**0**	**0**	**0**	**0**	**4**	**1**	**2**	**4**	0	0	4	0	0	0	0	2	0	1	0	0
Add	0			4			7			4			0			2			1		
Code	0474021																				

3.1.3. Antibiotic sensitivity:

The antibiotic sensitivity test is demonstrated in the following table.

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Table 6 : results of antibiotic test

Antibiotic Discs	P. vulgaris
chloramphenicol (20/10mcg)	R (0 mm)
ampicillin (15 µg)	R (0 mm)
Nitrofurantoin (300µg)	R (0 mm)
Gentamycin (10µg)	S (16 mm)
nalidixic acid (30mcg)	I (15 mm)

S: Sensitive R: Resistant I: Intermediate

4.DISCUSSION:

Some studies previously, described P. vulgaris infections in fish, (Okaeme, 1989) reported that P. vulgaris among other bacteria were the main cause of mortalities in hatcheries and outdoor ponds of tilapias and sharptooth catfish. P. vulgaris was also isolated from external ulceration of freshwater Channa punctatus (Mandal *et al.*, 2002). Clinical examination of infected fish showed that scales detachment, rejected pectora fin , haemorrhage in the body surface and congested gills are not quite different than those mentioned by (Ahmed & Elkamel, 2006).

Internally, there were congested kidney and spleen, Intestine of fish was filled with bloody serous fluid, This is similar to what he mentioned by (GALAL SAAD EL-DEEN, 2013). Bacteriological examination, the dominant isolates were from liver, kidney and spleen who agreed with (GALAL SAAD EL-DEEN, 2013) The phenotypic and culture characters of the colonies and the staining properties of the bacteria isolated from Nile tilapia O. niloticus suggested that the suspected isolates are P.vulgaris as was described by Austin and Austin (2007). Also the biochemical characters of P.vulgaris were agreed with finding of (Elgohary et al., 2020 In our study, P.vulgaris showed sensitivity to Gentamycin that agree with what mentioned by (Mordi & Momoh, 2009) and ciprofloxacin that agree with what mentioned by (Fam et al., 2013) and resistant to chloramphenicol and ampicillin and Nitrofurantoin, This was consistent with what he had concluded (Bilal et al., 2019) and (Lazm, Jebur, & Alomashi, 2018). In our current study, it was observed that unfavorable environmental conditions such as high

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temperature, the presence of suspended and decomposing organic materials, and the lack of dissolved oxygen in the water all led to an increase in the infection (virulence) and mortality rates, which is consistent with what was mentioned by (Bulbul Ali & Mishra, 2022)and (Mehta, 2017) and (Ikeogu, Nsofor, & Ikpeze, 2010).

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