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PROTEUS VULGARIS, AN EMERGING FISH PATHOGEN IN EGYPT

(With 4 Tables and 5 Figures)

By

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ميكروب البروتياس فولجارز حديث الظهور فى الاسماك بجمهوريه مصر العربيه

شعبان محمد احمد ، احمد عبد الهادى الكامل

الهدف من هذه الدراسه هو اجراء دراسه على اصـــابات اســماك العائلـــه القطيـــه النيليـــه (القراميط) فى محافظه اسيوط ج م ع بميكروب البروتياس فولجارز. تم فحـــص النتـــائج الاكلنيكيه و تشريح الاسماك المصابه و ايضا حساب معدل الاصابه الموسمى لها بالإضـــافه االى دراسه مقدره الميكروب على احداث التغيرات المرضيه.اجريت هذه الدراسه على عدد ١٢٠ سمكه من اسماك القرموط التى ظهرت عليها اصابات جلديه واعراض التسمم الدموى فى خلال عام ٢٠٠٥ وكانت نسبه الاصابه بميكروب البروتياس ١٩ % فقط. تم التعـــرف على الميكروب عن طريق اشكال المستعمرات وسلوك النمو الخلوي على مختلـــف انـــواع اوساط التغذيه الصناعيه و كذلك الفحص المجهرى والتفاعلات البايوكميائيه. بدراسه معـــدل الاصابه فى المواسم المختلفه اتضح ان الاصابه بالميكروب تبدأ فى الزياده فى فصل الربيع وتصل الى اقصاها فى فصل الصيف ثم تختفى فى الشـــتاء. بـــأجراء التجـــارب المعمليـــه بأستخدام العتره K93PV المعزوله من كلى الاسماك المصابه وجـــد ان الجرعـــه النصـــف مميته فى اسماك العائله القطيه (القراميط) تساوى $١,٢٥\times١٠^{٧}$ cfu/ ١٠٠ جرام اذا ما تـــم الحقن عن طريق العضلات واظهرت نتائج الفحص الاكلينيكى والتشريحى للاسماك المحقونه معمليا اصابات جلديه وفى بعض الاحيان حالات تسمم دموى وهى اكثر الاعراض ظهـــورا ومصاحبه لميكروب البروتياس فولجارز.

SUMMARY

The aim of this study was to investigate *Proteus vulgaris* infections in sharptooth catfish, *Clarias gariepinus*, in Assiut, Egypt. Clinical and postmortem findings of infected fish and seasonal prevalence of infection were investigated. Pathogenicity of *Proteus vulgaris* was also assessed. Out of 120 fish showing skin lesions and signs of septicemia examined over year 2005, only 1.58 (%) fish were infected with *Proteus*

vulgaris. Bacteria identification was based on colony morphology and culture behavior on various media, microscopic examination, biochemical tests and carbohydrate fermentation. Strain K93PV isolated from kidneys of infected fish was used throughout this study. Seasonal prevalence of *Proteus vulgaris* infections increased over spring and reached maximum in summer. Infection was not recorded in winter. Lethal dose 50 (LD_{50}) of *Proteus vulgaris* in sharptooth catfish was $1.25X10^7$cfu/100g fish body weight intramuscularly injected. Clinical and postmortem examination of experimentally infected fish revealed that skin lesions and sometimes generalized septicemia are the predominant signs associated with *Proteus vulgaris* infection.

Key words: *Proteus vulgaris, clarias gariepinus*

INTRODUCTION

Commercial farming of sharptooth catfish, *Clarias gariepinus*, has significantly increased in Upper Egypt over the past few years. Sharptooth catfish is widely accepted by consumers in Upper Egypt as a relatively cheaper choice of fish protein.

Many communities discharge their waste water into convenient rivers, lakes, or estuaries in spite of the fact that such material contains bacteria capable of causing human diseases. Unfortunately little consideration has been given to the possibility that fish exposed to such contaminated water may develop active infection and diseases. Pathogenic bacteria associated with the digestive tract of man and other mammals have been isolated from many varieties of fish (Abdel-Rahman *et al.*, 2004; Janssen and Meyers, 1968). Many members of *Enterobacteriaceae* can establish active infection in fish and persist for several weeks or longer (Lyayman, 1966).

Although *Proteus vulgaris* is considered mainly a human pathogen, it was reported to produce active infections in fish. Pervious 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, 1987) and mortalities in sharptooth catfish and tilapia (Okaeme, 1989). Furthermore, in a previous study conducted in our lab, *P. vulgaris* has been isolated from tissues of fish grown in ponds contaminated with un-treated chicken manure (Abdel-Rahman *et al.*, 2004).

Unfortunately, scarce data present on *Proteus vulgaris* infections in fish in Egypt. In the present study, prevalence and seasonal prevalence of *Proteus vulgaris* infections in sharptooth catfish have been investigated over one year. Clinical and postmortem sings and pathogenicity of *Proteus vulgaris* were also studied.

MATERIALS and METHODS

Seasonal prevalence and survey:

A total of 1200 alive sharptooth catfish, *Clarias gariepinus*, were collected from the small tributaries of El-Ibrahemia canal, Assiut City over a calendar year from January to December 2005 (100 fish/month). Out of the collected samples, 120 fish (10 fish/month) showing signs of infection were selected for thorough examination. The body weight of selected fish ranged from 300 to 350 g with total length of 30-34 cm.

Clinical and Bacteriological Examination of Samples:

Fish were examined for any apparent clinical signs or lesions according to Stoskopf (1993). Bacterial sampling were taken from both kidneys, liver and intestinal content on Cefsulodin Iragasan Novobioc (CIN) agar base supplemented with Yersinia selective supply (Biolife, Milno, Italy). Colonies were purified on brain heart infusion (BHI) agar (Biolife)

Bacterial identification:

Bacterial isolates were identified by colony morphology and cultural behavior on brain heart infusion (BHI) agar, trypticase soya agar (TSA) and salmonella shigella agar (SS agar), microscopic examination (Gram stain and motility test), biochemical tests that include oxidase, indole, Voges Proskauer, methyl red, urease and dnase activities, citrate utilization, triple suger iron reaction (TSI) and carbohydrate fermentation (arabinose, dulcitol, glucose, galactose, lactose, maltose, raffinose, salicin, sorbitol, and sucrose) according to Cowan *et al.* (1975); Cruickshank *et al.* (1975), Farmer and Mcwhorter (1984) and Brenner (1984).

Pathogenicity of *Proteus vulgaris* to *Clarias gariepinus*

A- Fish:

Apparently healthy sharptooth catfish with an average body weight of 100±5 g and total length as 19±1 cm were obtained from ponds of cultured sharptooth at Minia Governorate. Fish were acclimated to laboratory conditions for 2 weeks according to Ellsaesser and Clem (1986).

B- Bacterial strains:

All bacteria strains suspected to be *Proteus vulgaris* was kept in BHI broth with 15% glycerol (El-Gomhurrhia, Cairo, Egypt) at -20°C for further investigations. A bacterial strain isolated from kidney of infected fish and identified as *Proteus vulgaris* (K93PV) was passed through sharptooth catfish three times and used for determination of LD_{50} and pathogenicity experiments. Strain K93PV was grown in BHI broth at 28°C for 12 hours to mid-log phase (optical density of 1.2 at 600nm wave length) to be diluted and used for experimental infection.

C- Bacterial count:

Using optical density values at wave length of 600nm and standard plate count method (Elkamel *et al.*, 2003), colony forming units (cfu) counts in bacterial suspensions were determined. Counts were determined on BHI with 4% agar to inhibit swarming activities of *Proteus vulgaris*. Three concentrations of strain K93PV were made in distilled water as $1.25X10^7$, $2.5X10^7$ and $5X10^7$cfu/ml.

D- Determination of LD_{50}:

Sharptooth catfish weighing 100±5 grams were divided into nine groups where each group have four fish. All fish of each group were injected either intramuscularly (I/M) or intraperitoneally (I/P) with 1 ml of one bacterial concentration or distilled water as shown in next table.

Bacterial concentration	Route	No. of injected fish
$5X10^7$ cfu	IM	4
	IP	4
$2.5X10^7$ cfu	IM	4
	IP	4
$1.25X10^7$ cfu	IM	4
	IP	4
Control (distilled water)	IM	4
	IP	4
	Un-injected	4

Mortalities were recorded daily for four days. Moribund fish were bacteriologically examined to isolate the predominant bacteria from the internal organs and site of lesions. By the end of the forth day all fish alive were euthanized and examined. The whole experiment was repeated three times.

E-Experimental infection:

Sharptooth catfish weighing 100±5 grams were divided into three groups where each group have five fish. One ml of a bacterial suspension $1X10^7$ cfu/ml was I/M injected in all fish of one group. Two

groups (injected and non-injected) acted as control, where a group was I/M injected with 1ml of distilled water and the other one remained un-injected. Clinical signs and mortalities were recorded daily for 10 days. Alive and freshly dead fish were bacteriologically examined to isolate the predominant bacteria from the internal organs and site of lesions. The whole experiment was repeated three times.

RESULTS

Clinical Examination

A total of 120 fish showing signs of septicemia and/or skin lesions were selected after clinical examination of the collected sharptooth catfish, *Clarias gariepinus*. Fish showed several ulcerative lesions on body surface were noticed. Also, petechial haemorrhages were widely spread in many parts of the body particularly in mouth region, body surface and at the base of fins with fin rot. There were exophthalmia and corneal opacity either in one or both eyes in some of the examined fish. Liver was congested in some cases, while was pale in other cases. Petechial haemorrhages were observed on the liver surface and focal haemorrhages were also observed on the edge of liver. Gall bladder was enlarged and filled with bile. Spleen and kidney were congested and enlarged. Intestine was inflamed and blood vessels were congested.

Bacteriological examination

Bacterial examination resulted in isolation of 170 strains from liver, kidney and intestine of the 120 fish showing clinical signs of infection. Primary isolation was done on CIN agar. Based on cultural and morphological characters and swarming activities, 21 strains were suspected to be *Proteus sp*. Colonies grew well on CIN agar with red center that is surrounded by clear zone. On SS agar colonies were whitish or colorless with or without dark central spot. BHI and TSA agar showed highly swarming colonies that could be inhibited when agar concentration reaches 4% in media. Colonies on BHI with 4% agar media were thin, colorless and transparent.

Bacteriological identification

Out of 170 strains isolated from liver, kidney and intestine of the selected fish examined, 21 strains (12.35%) were suspected to be *P. vulgaris* according to morphological characters and biochemical identification. Microscopically, all suspected *P. vulgaris* strains were motile, Gram-negative rods ranged from short coccobacilli to long

filaments. Biochemical characters of the suspected 21 strains strongly suggest that those strains are *P. vulgaris* (Table 1).

Seasonal susceptibility and survey

Out of the fish collected, *P. vulgaris* were the predominant isolate in 19 fish (1.58%) suggesting active infection with *P. vulgaris*. In 2 cases (0.17%) of proteus infection, more than one strain of *P. vulgaris* were isolated form the same fish case. Seasonal susceptibility of *P. vulgaris* infection in sharptooth catfish is presented in Table 2 and organ susceptibility is presented in Table (3).

Determination of LD_{50}

Proteus vulgaris strain K93PV proved to be pathogenic to sharptooth catfish when injected I/M. Within 96 hours post infection half of the fish injected with a dose of $1.25X10^7$ cfu/100g fish body weight died. On the other hand, $2.5X10^7$ and $5X10^7$ cfu/100g fish body weight doses were highly lethal killing almost all fish within the first 4 days. Interestingly, I/P injection of fish even with the highest dose ($5X10^7$ cfu/100g fish body weight) did not lead to death of any fish but only one. *P. vulgaris* was re-isolated in pure culture from site of injection and internal organs of all I/M injected fish. Althoug *P. vulgaris* was re-isolated from internal organs of all I/P injected fish, only one fish died.

Experimental infection

Experimentally infected fish were sluggish in movement and easily caught by hand. The mucus which covers the body surface is increased. The most prominent signs were in the form of severe local reactions at site of injection (Fig. 1). It begins with severe local ischemia and paleness of the skin. Ulcers with a thin reddish hyperemic area surrounding a wide, white pale center was then developed (Fig. 2). Abscesses may develop and tissues become very friable. Dead tissues in the center may slough creating well demarked open ulcers with the underlying tissues become exposed (Fig. 3). In some cases, signs of generalized septicemia were observed. Petechial haemorrhages on the body surface particularly appeared at the base of fin and on the skin. Post-mortem examination showed that tissues at site of injection are severely inflamed and friable. Liver was congested in addition to petechial and focal haemorrhage on the surface and edge of the liver (Fig. 4). Gall bladder was enlarged and distended with bile. Kidneys were congested and enlarged. Congestion of all internal organs and blood vessels of gastrointestinal tract was observed (Fig.4). Spleen was darker than normal and larger in size with rounded edges (Fig.5). Two fish died at the fifth day post injection.

Table 1: Characters of 21 strains that are suspected to be *Proteus vulgaris*.

Test		*P. vulgaris* suspected strains
Gram stain		-
Motility		21/0
Oxidase		0/21
Vogus Proskauer		0/21
Indole		21/0
Methyl red		21/0
TSI	H_2S	21/0
	Gas	21/0
	K/A	2/19
Urease		21/0
Simmon's citrate		2/19
DNAse		0/21
Arbanios		-
Dulcitol		-
Glucose		+
Galactose		-
Lactose		-
Maltose		-
Rafinose		-
Salicin		+
Sorbitol		-
Sucrose		-

(+) positive (-) negative K/A Alkaline/Acid n=21

Table 2: Seasonal susceptibility of *Clarias gariepinus* to *Proteus vulgaris* infection.

Season	No. of fish collected	No. of fish infected	Percent of infection
Winter	300	0	0
Spring	300	7	2.33
Summer	300	10	3.33
Autumn	300	2	0.33

Table 3: Organ susceptibility of *Clarias gariepinus* to *Proteus vulgaris* infection.

Organ	Infected fish (n=19)		Isolated strains (n=21)	
	No.	%	No.	%
Liver	4	21.1	5	23.81
Kidney	7	36.8	7	33.33
Intestine	8	42.1	9	42.86

Table 4: Determination of LD_{50}. Data are from one of three experiments.

Dose	Injection	No. of fish died post infection				No. of fish survived
		Day 1	Day 2	Day 3	Day 4	
$5X10^7$ cfu	IM	2	2	-	-	0
	IP	0	0	1	0	3
$2.5X10^7$ cfu	IM	1	2	0	0	1
	IP	0	0	0	0	4
$1.25X10^7$ cfu	IM	0	1	1	0	2
	IP	0	0	0	0	4
Control (distilled water)	IM	0	0	0	0	4
	IP	0	0	0	0	4
	Un-injected	0	0	0	0	4

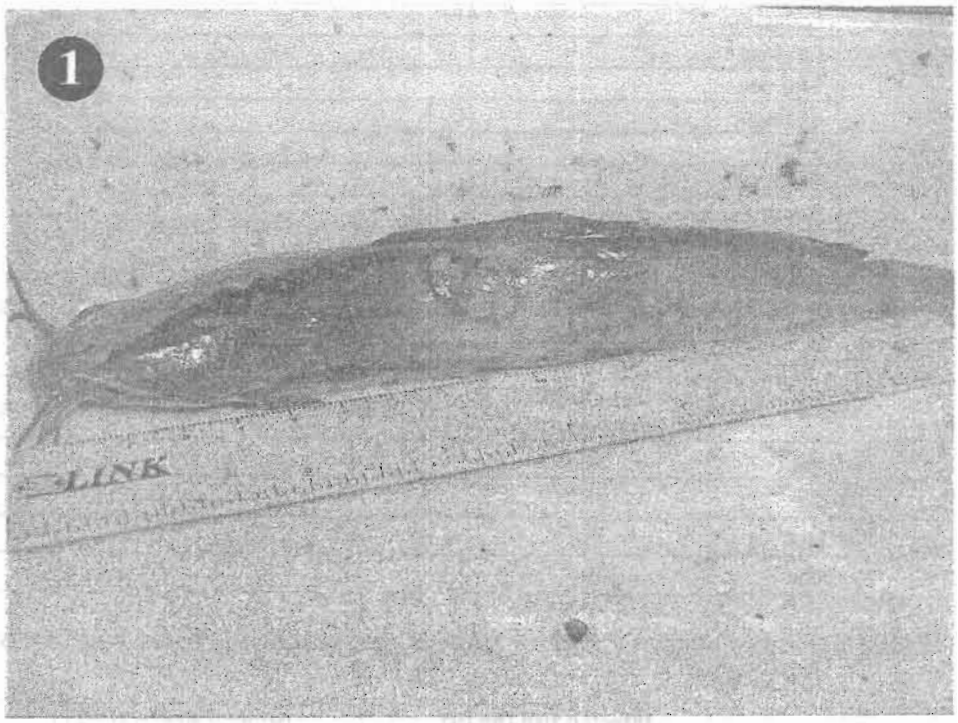

Fig. 1: Sharptooth catfish, *Clarias gariepinus*, 24 hours post intramuscular injection with *Proteus vulgaris* ($1X10^7$ cfu/100g body weight) showing severe local reactions at site of injection.

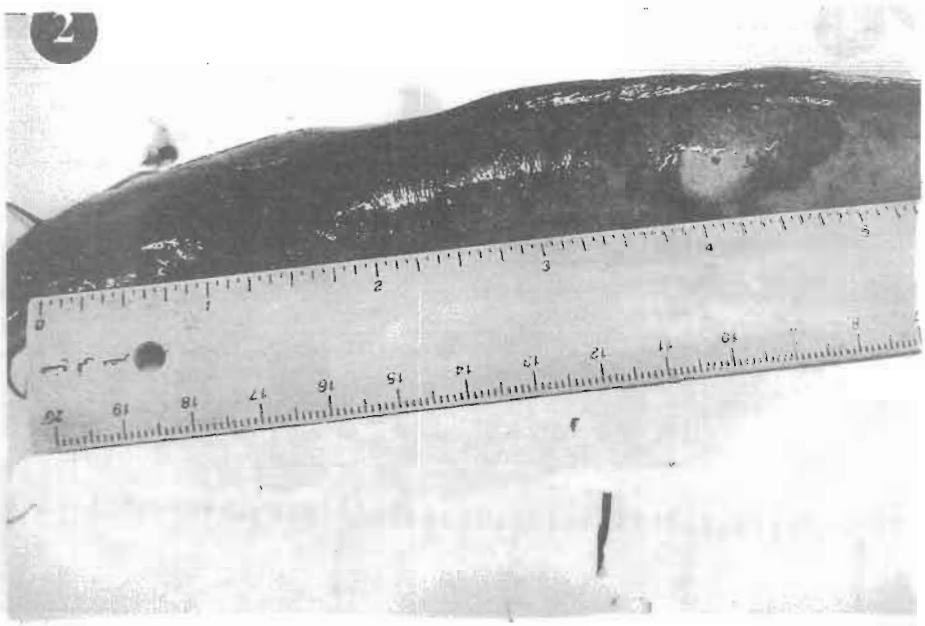

Fig. 2: Sharptooth catfish, *Clarias gariepinus*, 48 hours post intramuscular injection with *Proteus vulgaris* ($1X10^7$ cfu/100g body weight) showing skin ulcers.

Fig. 3: Sharptooth catfish, *Clarias gariepinus*, 72 hours post intramuscular injection with *Proteus vulgaris* ($1X10^7$ cfu/100g body weight) showing open ulcers and sloughing of dead tissues.

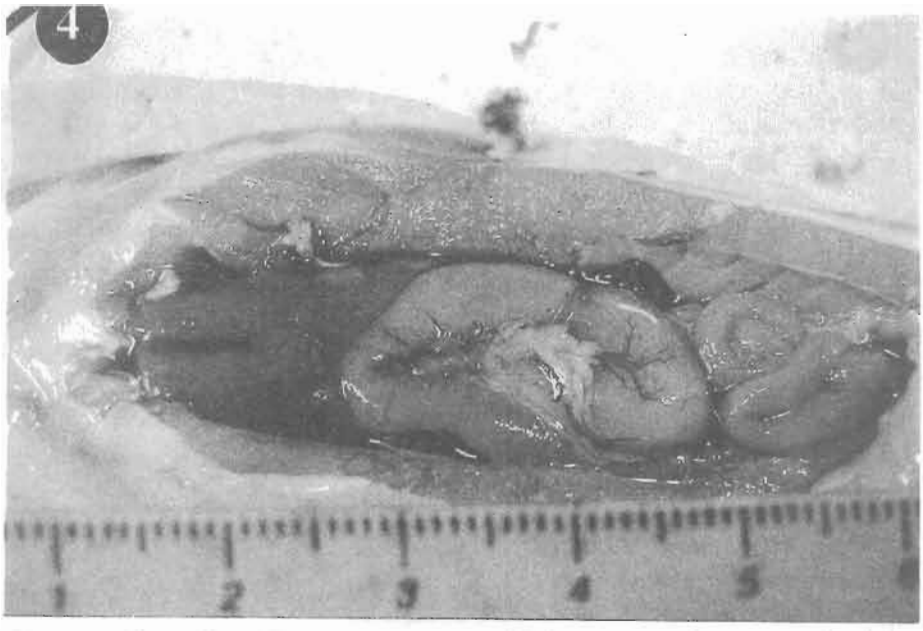

Fig. 4: Sharptooth catfish, *Clarias gariepinus*, 72 hours post intramuscular injection with *Proteus vulgaris* ($1X10^7$ cfu/100g body weight) showing congestion of liver and intestine.

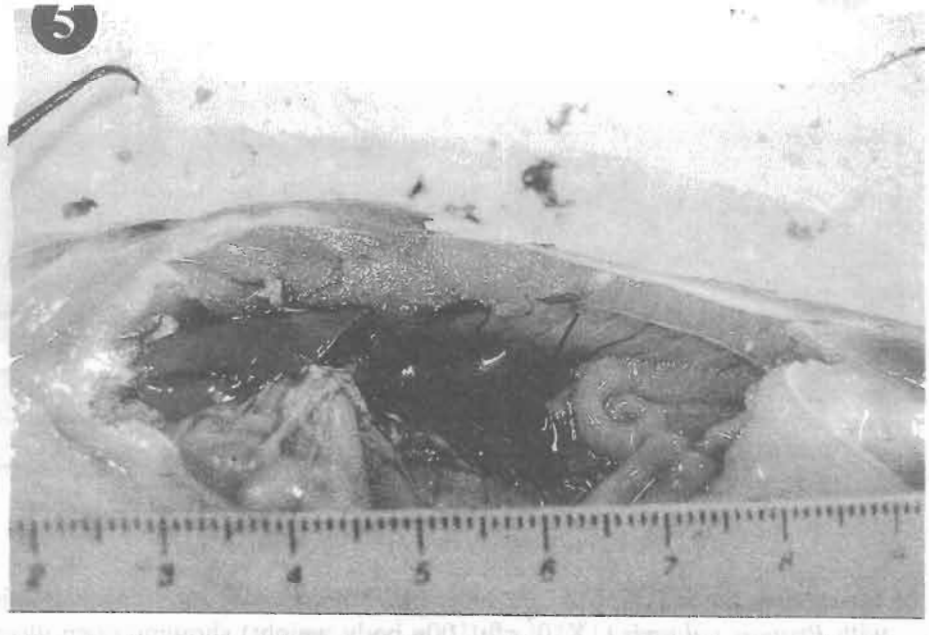

Fig. 5: Sharptooth catfish, *Clarias gariepinus*, 72 hours post intramuscular injection with *Proteus vulgaris* ($1X10^7$ cfu/100g body weight) showing enlarged spleen with rounded edges (arrow).

DISCUSSION

Present study revealed that *Proteus vulgaris* infection of sharptooth catfish, *C. gariepinus*, is an emerging disease in Egypt. A pervious study that reported the isolation of *Proteus spp.* from fish was the driving force behind conducting this investigation (Abdel-Rahman *et al.*, 2004). Those findings lead to a large-scale study to investigate the seasonal prevalence, clinical signs and post mortem lesions of *P. vulgaris* infection in sharptooth catfish. Also, LD_{50} of *P. vulgaris* was determined.

Most of studies that investigated *P. vulgaris* contamination of fish focused on water quality issues or the role of fish in transmitting such pathogen to human and food poisoning (Okpokwasili and Alapiki, 1990; and Youssef *et al.*, 1992), but overlooked the impact or the risk on fish health itself. Some studies previously, however, described *P. vulgaris* infections in fish, and this study is the first to characterize infections of sharptooth catfish in Egypt.

Thomas *et al.* (1983) have shown that fish grown in ponds containing waste water accumulate *Enterobacteriaceae*, including *Proteus spp.*, that penetrate into the muscle tissues of 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). Based on serological evidences, a study has concluded that fish exposed to enteric bacteria that are mainly human pathogens may become actively infected with such pathogens (Janssen and Meyers, 1968).

Clinical examination of infected fish showed that skin lesions and general septicemia are the main signs of suspected naturally infected fish. Septicemia may be due to generalized infection where liver and most internal organs become congested. Spleen and gall bladder become also enlarged. Badran *et al.* (1994) concluded that clinical signs of fish infected with human *Enterobacteriaceae*, which are not considered classical fish pathogens bacteria, are not quite different than those of classical fish pathogens. Okaeme (1989) stated that signs of fish infected with *P. vulgaris* and other bacteria are ulceration and necrotic lesions of skin, abdominal dropsy and haemorrhagic septicemia. Furthermore, Skin ulceration and lesions were reported in an infection with *P. vulgaris* and other bacteria that were complicated with a parasitic copepod in akame (Muroga, 1979).

Bacteriological sampling of selected fish was done from internal organs but not from surface or skin of fish. Isolation of *P. vulgaris* from internal organs suggests a condition of generalized infection rather than a surface contamination. Accurate identification of bacterial strains suspected to be *P. vulgaris* was done through investigating 20 morphological, growth characters and biochemical characters. Strain K93PV isolated in June 2005 from kidneys of a fish showing signs of septicemia and skin lesion was identified as *P. vulgaris* and used thorough out this study for determination of LD_{50} and pathogenicity study. Primary isolation was done on CIN agar which is a selective media for Yersinia.

Prevalence of infection was 1.58% of all fish collected over one year. The prevalence of *P. vulgaris* infection increased in spring and summer, while decreased in autumn and disappeared in winter. Results suggest that water temperature has a great effect on prevalence of infection. Prevalence of infection is highest when the water temperature is highest in summer when it matches the optimum temperature for growth of *P. vulgaris*. Although water temperature in autumn is more suitable for *P. vulgaris* than that in spring, the infections prevalence was higher in spring. These results may be due to the fact that the immune system of fish in spring would be still recovering from the cold winter and not fully protective against infections. On the other hand, organ susceptibility study showed that *P. vulgaris* is mainly isolated from the fish intestine (42.1%). These findings were expected as *P. vulgaris* is a member of family *Enterobacteriaceae* that inhabit intestinal tract of vertebrates. Kidneys come second in organ susceptibility where *P. vulgaris* was isolated from 7 (36.8%) cases. Isolation of from liver occurred in 4 (21.1%) cases.

Results revealed that $1.25x10^7$cfu/100g fish body weight is the LD_{50} dose when I/M injected. The two higher doses proved to be highly lethal to fish when I/M injected. An earlier study concluded that LD_{50} of *P. vulgaris* in carp fingerlings and yearlings was $0.48X10^5$ cfu/fish (Ramaiah and Manohar, 1988). The differences in LD_{50} doses from previous study may be due to species, lifestyle, or age differences. Sharptooth catfish may be more naturally resistant to *P. vulgaris* than carps. Because of its lifestyle, sharptooth catfish may be exposed to bacterial agents more than other species of fish which might results in developing of some level of resistant to bacterial infections. In addition, carps used in such study were younger than sharptooth catfish used in present study.

Surprisingly, I/P injection of *P. vulgaris* in the same doses used with I/M injection did not lead to killing of injected fish but one. Only one fish died after I/P injection with the highest dose of *P. vulgaris* that could be either due to stress or overwhelming of the immune system with bacteria. Although *P. vulgaris* could be isolated from internal organs of fish up to four days post I/P injection, no major external clinical signs were obvious. Those results suggest that fish I/P injected with *P. vulgaris* can either overcome infection or completely recover.

Results of present study could give clues about route of natural infection with *P. vulgaris* in sharptooth catfish. Skin injuries would be a major route of infection where bacteria penetrate though compromised skin giving a strong local reaction. Fish live in small tributaries of El-Ibrahimiah canal are subject to sewage and waste water. Bacteria go through skin might remain in the underlying tissues and multiply to overwhelm the immune system causing a generalized infection at terminal stages. Alternatively, *P. vulgaris*, a normal inhabitant of fish intestine, might seize stress conditions that fish face to cause septicemia and generalized infection. In both cases, *P. vulgaris* may require stress conditions to induce an active infection in fish. Although, those bacteria are not considered classical fish pathogens, yet adverse environmental conditions and poor water quality render fish more susceptible to infection with those bacteria (Badran *et al.*, 1994).

Further investigations should be done to study the epidemiology of *P. vulgaris* in fish.

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