

Animal Health Research Institute (AHRI)
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ISOLATION AND IDENTIFICATION OF ENTEROBACTERIACEAE FROM FRESHWATER GRAYFISH (*PROCAMBARUS CLARKII*)

(With 4 Tables and 2 Figures)

By

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عزل وتصنيف بكتيريا الأمعاء السالبة الجرام (الأنتيرو بكتيرياس)
فى أستاكوزا المياه العذبة

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الهدف من هذه الدراسة هو إجراء دراسة على وجود بكتيريا الأمعاء السالبة الجرام فى أستاكوزا المياه العذبة (أستاكوزا المستقعات الحمراء). وقد تم فحص النتائج الأكلينيكية وتشريح الأستاكوزا وأيضاً عزل الميكروبات وتحديد نسبة الإصابة. أجريت هذه الدراسة على عدد ٥٠ من أستاكوزا المياه العذبة ووضعها تحت الفحص الظاهري والتشريح المرضى والفحص البكتيري. وكان من نتيجة الفحص البكتيريولوجي عزل ١٥٠ عترة من الغدة الهضمية والسائل الدموي الليمفاوي والعضلات والأمعاء تتفق بدرجة كبيرة مع مجموعة الأنتيرو بكتيرياس. وقد تم التعرف على الميكروبات عن طريق أشكال المستعمرات وسلوك النمو الخلوي على مختلف أوساط التغذية الصناعية وكذلك الفحص المجهرى والتفاعلات البيوكيميائية والتي تم تصنيفهم إلى ١٦ عترة إيدوردسيلا تاردا ، ١٠ عترات بروتيس فالجارز ، ٦٢ عترة بروتيس ميرباليزا ، ٣٢ عترة سالمونيلا و ٣٠ عترة لم يتم التوصل إلى تصنيف لهم. وقد ثبت من خلال النتائج أن الأمعاء هي أكثر الأعضاء ملائمة لعزل ميكروب الإيدوردسيلا تاردا ، بينما العضلات هي أكثر ملائمة لعزل ميكروب سالمونيلا والبروتياس فالجارز غير أن الغدة الهضمية هي أكثر ملائمة لعزل ميكروب البروتياس ميرباليز.

SUMMARY

The aim of this study was to investigate Enterobacteriaceae infection in freshwater crayfish (red swamp crayfish), *Procambarus clarkii*, clinical, postmortem findings of examined crayfish, bacteriological examination, rate of infection and organ susceptibility were investigated. 50 freshwater crayfish were subjected to clinical, postmortem and bacteriological examination. Bacteriological examination revealed to isolation of 150 strains from digestive gland, haemolymph, musculature and intestine which are close to morphological and cultural characters to

Enterobacteriaceae. Bacteria identification was based on colony morphology and culture behavior on various media, microscopic examination, biochemical tests and carbohydrate fermentation. 150 strains were identified as *Edwardsiella tarda* (16), *Proteus vulgaris* (10), *Proteus mirabilis* (62), *Salmonella* (32) and 30 isolates were not identified. Bacteriological examination revealed the best organ for isolation of *E. tarda* is intestine, while musculature is the best site for isolation of *Proteus vulgaris*, and *Salmonella*. Moreover, hepatopancrease is the best organ for isolation of *Proteus mirabilis*.

Key words: *Enterobacteriaceae*, *Crayfish*.

INTRODUCTION

The redswamp crayfish, *Procambarus clarkia* is an autochthonous species from the Northeast of Mexico and South Central US. Hobbs *et al.* (1989) which was introduced worldwide and has become the dominant freshwater crayfish in almost all area it occupies. Henttonen and Huner (1999).

Introduction of *P. clarkii* to River Nile, Egypt, was through a commercial aquaculture in Giza (Manial-Shetha), in the early 1980 when first immigrants of this species were introduced from USA and the project was shortly terminated due to administrative failure. *P. clarkii* can be use as a source of animal protein for Egyptian people, many of them suffering from mal-nutrition, where the crayfish muscles contain higher values of protein (58.6 g/100g of female, and 62.6 g/100g for male), while the dried carapace can also act as a rich constituent of poultry food Mona *et al.* (1999). Enterobacteriaceae are a very large family of some 25 genera and more than 100 species of facultatively anaerobic and gram-negative rods. Some species from this family are important pathogens of man, animals and fish Shoots and Bullock (1975). Enterobacteriaceae species either *Edwardsiella tarda*, *Proteus vulgaris*, *Proteus mirabilis* and *Yersinia* were considered of the most important bacterial microorganisms, that causes severe economic losses among fish Farms in many countries as it was reported in most of world countries as, North America, Japans, Taiwan, Thiland and Africa. Bragg (1991), Durborow *et al.* (1991), Francis *et al.* (1993) and Baya *et al.* (1997).

Unfortunately, scarce data present on Enterobacteriaceae in freshwater crayfish in Egypt. This work was planned to fulfill the following objectives, full description of clinical signs and postmortem lesions of naturally infected crayfish. Isolation and identification of

different strains of Enterobacteriaceae group with giving light on percentage of infection and organ susceptibility.

MATERIALS and METHODS

Crayfish:

A total of 50 alive crayfish *P. clarkii*, were collected from the small tributaries of El-Ibrahemia cannal and River Nile, Assiut city. A total length of examined crayfish ranged from 9-16 cm. crayfish were transported to animal health Research Institute in Assiut where clinical and bacteriological examination were conduct.

Clinical and bacteriological examination of crayfish

Crayfish were examined for any apparent clinical signs or lesions according to Melba *et al.* (2001). Bacterial sampling were taken from both haemolymph, hepatopancrease, Musculature and intestinal content on salmonella shigella agar (SS agar) (Bio life). Colonies were purified on brain heart infusion (BHI) agar (Bio life).

Bacterial identification

Bacterial isolates were identified by colony morphology and cultural behavior on brain heart infusion agar (BHI), Trypticase soya agar (TSA) and Salmonella shigella agar (SS agar), microscopic examination (Gram stain and motility test), biochemical tests that include oxidase, catalase, indole, methyl red, voges proskauer, citrate utilization, triple sugar iron reaction (TSI), urease and carbohydrate fermentation (Glucose and sucrose) according to Cruickshank *et al.* (1975), Hawke *et al.* (1981), Farmer and Mcwhorter (1984), Brenner (1984) and Whitman (2004)

RESULTS

Clinical Examination:

A total of 50 freshwater crayfish showing black spot on the back of the abdomen (Fig. 1), legs, cheliped and tail fin, haemorrhages on hepatopancrease (Fig. 2).

Bacteriological examination:

Bacterial examination resulted in isolation of 150 strains from hepatopancrease, Musculature, intestine and haemolymph of 50 freshwater crayfish. Primary isolation was done on SS agar. Isolates were identified according to culture and morphological characters as Enterobacteriaceae. 16 strains were suspected to be *Edwardsiella tarda*. Colonies on SS agar were small, black centers to predominant black colonies. Colonies on BHI and TSA agar were small, round-raised

transparent colonies. 10 strains were suspected to be *Proteus vulgaris* and 62 strains were suspected to be *Proteus mirabilis*. Colonies grew well on SS agar; colonies were whitish or colorless with or without dark center spot. Colonies on BHI, TSA were thin, colorless and transparent. 32 strains were suspected to be *Salmonella*. *Salmonella* colonies on SS agar gave flatter and colourless or opaque colonies with small, black center. *Salmonella* colonies on BHI and TSA give grey-white, moist, smooth colonies.

Bacteriological identification:

Microscopically, all strains (150) were motile, Gram-negative rods ranged from short coccobacilli to long filaments and oxidase negative. Biochemical characters of isolates are recorded in Table (1).

Frequent distribution of the isolated pathogens

Isolates were identified according to culture, morphology and biochemical characters as *Edwardsiella tarda* (16 isolates), *Proteus vulgaris* (10 isolates), *Proteus mirabilis* (62 isolates), *Salmonella spp.* (32 isolates) and 30 isolates were not identified. These results were demonstrated in Table (2).

Percentage of infection among crayfish:

Percentage of infection among freshwater crayfish examined (50) revealed that 12 crayfish (24%) were infected with *E. tarda*, 9 crayfish (18%) were infected with *P. vulgaris*. Moreover, number of crayfish infected with *P. mirabilis*, *Salmonella sp.* were 36 (72%), 22 (44%) respectively. These results are summarized in Table (3). While organ susceptibility of isolated strains were found in Table (4).

Table 1: Biochemical characters of isolated bacteria:

Test	<i>E. tarda</i>	<i>P. vulgaris</i>	<i>P. mirabilis</i>	<i>Salmonella</i>
N. of strain	16	10	62	32
Gram-stain	G-ve short rod bacilli			
Motility	+	+	+	+
Oxidase	-	-	-	-
Catalase	+	+	+	+
Indole	+	+	-	-
Methyl-red	+	+	+	+
Vogus-proskauer	-	-	-	-
Simmon's citrate	+	+	+	+
TSI H ₂ S	+	+	+	+
Gas	+	+	+	+
Urease	-	+	+	-
Glucose	+	+	+	+
Sucrose	+	-	-	-

+ positive

- negative

Table 2: Frequent distribution of the isolated bacteria (n= 150 strains).

Bacterial species	No. of strains	Percentage
<i>Edwardsiella tarda</i>	16	10.67%
<i>Proteus vulgaris</i>	10	6.67%
<i>Proteus mirabilis</i>	62	40.13%
<i>Salmonella spp.</i>	32	21.33%
Un-identified isolates	30	20%

Table 3: Percentage of infection in freshwater crayfish (50).

Disease	No. of infected crayfish	%
<i>Edwardsiella tarda</i>	12	24%
<i>P. vulgaris</i> infection	9	18%
<i>P. mirabilis</i> infection	36	72%
<i>Salmonella</i> infection	22	44%

Table 4: Organ susceptibility of isolated strains.

Bacteria spp.	DG		HL		M		I	
	No.	%	No.	%	No.	%	No.	%
<i>E. tarda</i>	2	12.5%	1	6.25	2	12.5	11	68.75
<i>P. mirabilis</i>	24	38.7	2	3.23	22	35.48	14	22.58
<i>P. vulgaris</i>	3	30	0	0	4	40	3	30
<i>Salmonella</i>	10	31.25	2	6.25	14	43.75	6	18.75

N.B. DG digestive gland
 HL Haemolymph
 M musculature
 I intestine

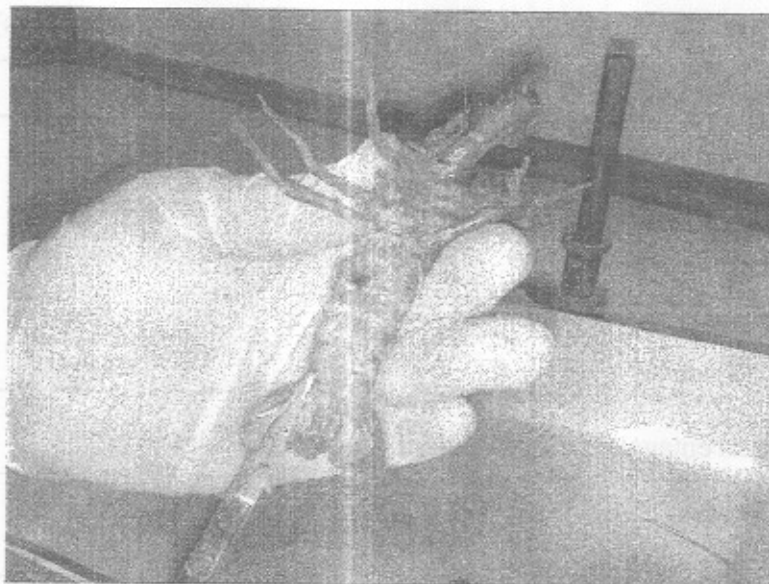


Fig. 1: Freshwater crayfish, *Procambarus clarkii* naturally infected with *Enterobacteriaceae* revealing black spot on abdomen.

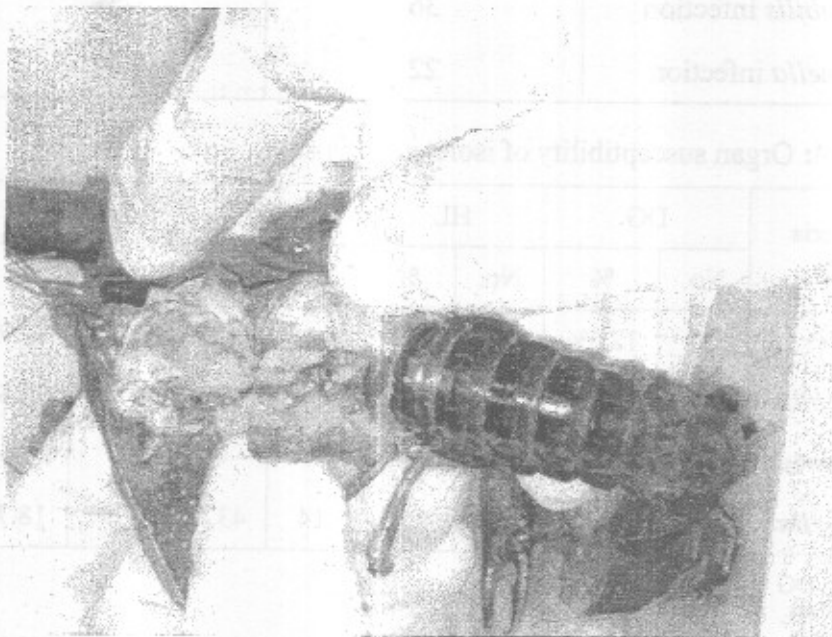


Fig. 2: Freshwater crayfish naturally infected with *Enterobacteriaceae* showing haemorrhage on digestive gland.

DISCUSSION

Freshwater crayfish, *Procambarus clarkii* which recently appeared in River Nile and its resources all over Egypt was bacteriological examination to isolate Enterobacteriaceae. Although Enterobacteriaceae species were successfully isolated from haemolymph, digestive gland, intestine, and musculature of freshwater crayfish examined in this study. No typical clinical signs of infection were evident. This results can be explained by Scott and Thune (1986), Webster (1995), Wong *et al.* (1995) and Madetoja and Jussila (1996) who reported, asymptomatic bacteremia have been reported in apparently healthy freshwater crayfish including *Astacus astacus*, *Chjerax albidus-destroyer*, *Cherax quadricarinatus* and *Procambarus clarkii*. Prevalence of asymptomatic bacteria was 100% in investigated apparently healthy *Procambarus clarkii*.

Black spot were appearing in the examined crayfish. This observation may be attributed to immune system of crayfish Soderhall and Ajaxon (1982). 150 strains of Enterobacteriaceae were isolated from examined freshwater crayfish. El-Sherry (2009) isolated 64 strains of Enterobacteriaceae from 48 freshwater crayfish. These results can be explained by Tubiashi *et al.* (1975) and Davis and Sizemore (1982) who reported the bacteria inhabited the ecosystem in which the crayfish live and were found in water and sediments, resident on the exoskeleton or in the gut. They enter the hemocoel through minor wounds, gastrointestinal tract and other different routes. Fish grown in ponds containing waste water accumulate Enterobacteriaceae, that penetrate into muscle tissue of fish Tomas *et al.* (1983). Otherwise, Baya *et al.* (1997) have reported fish living in river water that receives city sewage carry a significantly higher number of bacterial species in comparison with fish from less polluted water. 150 isolates of Enterobacteriaceae were identified according to culture, morphology and biochemical characters as *E. tarda* (16), *Proteus vulgaris* (10 strains), *Proteus mirabilis* (62 strains), *Salmonella* (32 strains) and (30 strains) non-identified strains. Results of biochemical tests were nearly similar to those reported by other investigation including, Farmer and Mcwhorter (1980), Grimont *et al.* (1980), Farmer (1995), Whitman (2004) and Ahmed and El-Kamel (2006). *Edwardsiella tarda* was isolated from freshwater crayfish. Wyatt *et al.* (1979) can isolate *E. tarda* from 100% examine crayfish. Result showed that *E. tarda* was isolated from intestine higher than from digestive gland, Haemolymph and musculature. This could be due to infection with *E. tarda* begin from intestine and spread to other visceral

organs. *Proteus vulgaris* (10) were isolated from crayfish. Toumanoff (1968) was isolated *P. vulgaris* from *Procambarus clarkii* and *Orconectes timosus*. *P. vulgaris* was isolated from musculature at higher rate than hepatopencrease, Haemolymph and intestine. These result nearly to Thomas *et al.* (1983) which have reported that fish grown in pond containing waste water accumulate Enterobacteriaceae, that penetrate into the muscle of fish. 32 strains salmonella was isolated and these result can be attributed to, fresh water crayfish are typical found in warm freshwater rivers, slowflowing water, reservoirs and irrigation system which are subject to sewage, waste water, mud and dead animals. *Salmonellae* are typical mesophilic bacteria. However, their main as water reservoirs, contaminated with human and animals excrete may harbour salmonella. In particular shellfish growing in contaminated water may accumulate salmonella and raw oysters (Ahmed, 1991). *Salmonella* has also been detected in several crustacean and molluscan product from Indian and Malaysia (D'Aoust, 2000). While Elmossalami and Emara (1999) reported that *Salmonella* could not be isolated from any of the examined *P. clarkii*.

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