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Detection of *Vibrio* Spp. In Water, Some Aquatic Insects and Fish Species in River Nile, Egypt

[1]M.A. Hassanain, [2]Nadia M. Lotfy, [3]Kadria N. Abdel Megeed, [4]Fagr Kh. Abdel-Gawad, [4]G.E. El Taweel and [4]S.M. Bassem

[1]Department of Zoonotic Diseases, National Research Center, Dokki, Giza, Egypt
[2]Department of Entomology, Faculty of Science, Ain Shams University, Cairo, Egypt
[3]Department of Parasitology and Animal Diseases, National Research Center, Dokki, Egypt
[4]Department of Water pollution Research, National Research Center, Dokki, Egypt

Abstract: Members of the genus *Vibrio* are Gram-negative motile bacteria that were naturally occurring, free-living inhabitants of marine and estuarine environments throughout the world. It was found that 425 total vibrios were isolated from some aquatic insects, some fish and water collected from River Nile at different polluted sites from which only 202 isolates were +ve *Vibrio* spp. when tested by PCR. Total vibrios were determined in water collected from different polluted sites. The average values of total vibrios (MPN/100 ml) recorded in water was 0, 1.0×10^4 and 2.7×10^3 in the main stream of River Nile before branches, mixed point of agriculture drain and the mixed point of wastewater, respectively. The average MPN/gm of total vibrios recorded in aquatic insects was 1.4×10^3, 1.7×10^5 and 1.3×10^5 in the main stream of River Nile before branches, the mixed point of agriculture drain and the mixed point of wastewater respectively. Also, total vibrios were determined in fish collected from River Nile from different polluted sites in 3 organs; muscle, liver and gills. Muscle did not contain any *Vibrio* spp. in the different sites. In the main stream of River Nile before branches, liver did not contain any *Vibrio* spp. The average MPN/gm values of total vibrios in liver isolated from fish collected from mixed of agriculture drain and the mixed point of wastewater were: 2.9×10^4 and 6.2×10^3, respectively. The average MPN/g values of total vibrios in gills isolated from fish collected from the main stream of River Nile, the mixed point of agriculture drain and the mixed point of wastewater were: 3.8×10^3, 1.0×10^4 and 2.5×10^4 respectively. It can be concluded that: water pollution affects the aquatic life and also aquatic insects and fish may be used as bioindicators of water zoonotic pathogens.

Key words: Aquatic Insects · Fish · Water Pollution · *Vibrio* spp. · PCR

INTRODUCTION

The River Nile constitutes is very important aquatic and wetland ecosystems and it supplies about 97% of Egypt's water reserves [1]. Pollution in the River Nile System (main stream Nile, drains and canals) increased in the past few decades because of increasing population, several new irrigated agriculture projects and other activities along the Nile. Different genera of pathogenic bacteria were detected in River Nile water at Beni-Suif area, Egypt [2]. A great variety of insects were carrier of pathogens [3]. At the beginning of this century Lemley and King [4] reported on an insect-bacteria bioindicator for assessing detrimental nutrient enrichment in wetlands. Mahilum *et al*. [5] detected *Wolbachia* infections in *Culex pipiens* complex mosquitoes from the upper Rhine Valley in Germany and Cebu City in Philippine. *V. cholerae* was isolated from the egg masses of *Chironomus* sp. in waste stabilization pond and it was found to be the cause of the egg mass destruction [6]. Waterborne pathogens are causative agents for many human diseases and their presence in water bodies poses a potential threat to the human population. Because of the risk of public outbreaks of disease, it is of utmost importance that communities be aware of potential risks associated with water contact, including the presence of frankly pathogenic bacteria.

Corresponding Author: N. Kadria Abdel Megeed, Department of Parasitology and Animal Diseases, National Research Center, Dokki, Giza, Egypt.

It has been estimated that 50 000 people die daily in the world as a result of water related disease [7]. Sewage effluents contain a wide range of human enteric pathogens, which may pose a health hazard to the exposed human population when they are discharged into natural waters [8]. Several pathogenic *Vibrio species* are known to be commonly associated with outbreaks of *Vibrio* infections due to consumption of food and water contaminated with human feces or sewage, raw fish and seafood or with exposure of skin lesion such as cuts, open wounds and abrasions to aquatic environments and marine animals [9]. The genus *Vibrio* includes more than 60 species other than *V. cholerae* which is harmful to man through ingestion of contaminated water. Moreover, *V. parahaemolyticus*, *V. mimicus* and *V. vulnificus* are food-poisoning bacteria which are frequently isolated from seawater and seafood [10]. The laboratory methods most often used to detect and identify the pathogenic *Vibrio* spp. relied on culture followed by conventional biochemical, serological and susceptibility testing. However, these methods were time consuming, labor and reagent intensive and usually didn't directly characterize the virulence factors associated with human and animal diseases.

The development of rapid and sensitive molecular techniques for the detection of *Vibrio species* would be useful for the surveillance of sporadic infections and management of major outbreaks. Comparative sequence analysis of the fts Z gene in the predominant *Vibrio species* that cause human disease revealed distinct alleles for each examined species, including *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. Light Upon extension (LUX) real-time PCR assays were developed to target these species-specific polymorphisms and were successful in rapidly differentiating the major pathogenic *Vibrio species*. Luminex liquid microsphere array technology was used to develop a comprehensive assay capable of simultaneously detecting *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. These assays permitted the identification of a presumptive *V. parahaemolyticus* isolate as *V. alginolyticus*, which was verified using additional molecular characterization [11]. Luan *et al.* [12] adopted MPN-PCR (most probable number-polymerase chain reaction) for rapid detection of the quantity of *V. parahaemolyticus* in seafood. *V. parahaemolyticus* in seafood could be quantitated by MPN statistics according to PCR products.

Therefore, the present study aims to determine of total *vibrios* in water, aquatic insects and fish from River Nile.

MATERIALS AND METHODS

Samples Collection and Identification

Water Sampling: Water samples were collected weekly from the study sites in River Nile during the period of investigation. Three different types of water were collected as follows; the main stream of River Nile was considered as control (reference point), River Nile mixed with agriculture drainage and River Nile mixed with wastewater drainage. Samples were collected from the middle of the river (30 cm deep from the surface) in a wide mouth, sterile glass bottles (double) one liter volume. Then samples were transferred to the laboratory at National Research Centre in ice box within 2-4 hours and analyzed for detection and enumeration of total vibrios.

Aquatic Insects and Fish Sampling and Identification: Aquatic insects and fish were collected monthly, for 12 months for detection of total vibrios. Aquatic insects were collected by sweeping the water with D-framed net, Short sweeps through water with the rim of the sieve just grazing the bottom of the stream, yields the best results with some species inhabiting shallow and vegetated area are collected by disturbing the bottom with a net and sweeping the net through the disturbed area to capture specimens [13]. Then samples were transferred in ice box to the laboratory for identification and bacterial examination. Fish were also collected from the same places as aquatic insects and transferred alive to the laboratory for bacterial examination tests. Collected fish species include Tilapia and catfish. Identification was mainly based on available keys [14]. For maximum certainty, determinations arrived through the use of keys that were compared with correctly identified specimens in Department of entomology, Faculty of Science, Ain Shams University.

Bacteriological Examination: The collected insects and fish samples were decontaminated externally by washing several times in dis. H_2O then in 70% ethanol. Fish were dissected and 3 organs were examined for bacterial contamination; liver, gills and muscles. One gram of sample tissue (insects and fish) was homogenized in 9 ml of 9% saline then used in the experiments [15].

Determination of total Vibrios by MPN-TCBS Method: One ml of water sample was transferred to 9 ml of 0.9% NaCl solution then the solution was diluted up to 10^5. One g of whole insect and fishes (muscles, liver and gills) were homogenized and then added to 9 ml of 0.9% NaCl solution, then the solution was diluted up to 10^5.

In case of samples collected from River Nile before branches; raw sample and 2 dilutions only were inoculated while samples collected from drains (agriculture and wastewater) up to dilutions were inoculated. One ml of each dilution was transferred to another five tubes of alkaline peptone broth using appropriate concentration of broth relative to sample volume. The tubes were incubated at 37°C for 6-8 hours in incubator (Gerhardt), then a loopful of each tube was streaked onto thiosulfate-citrate-bile salt-sucrose (TCBS) agar plate and these plates were incubated at 37°C for 18-24 hours. Suspected *V. cholerae* colonies appear in yellow color and small in diameter while green colonies were screened and examined for *V. parahaemolyticus* and *V. vulnificus* by PCR (5 random typical colonies). Typical colonies were collected and streaked on slants of tryptic soya agar (TSA) and stored at 4°C not more than one year [15].

Detection of *Vibrio* spp. by PCR: First samples were prepared for DNA extraction as follows: A loopful of each isolate was transferred to 5 ml Tryptic soya broth (TSB) tubes, incubated at 37°C for 18-24 hours, then streaked on nutrient agar and incubated at 37°C for 18-24 hours. Single colonies were picked into 50 ml TSB and incubated at 37°C for 18-24 hours, then centrifuged at 3700 g for 15 min at 4°C in cooling centrifuge (Mikro 22 R, Hettich-Zentriufugen). The supernatant was discarded and the pellet resuspended in Phosphate buffer saline (PBS) (pH: 7.2) twice then centrifuged at 3700 g for 15 min. The pellet was resuspended in 1000µl PBS, finally stored at -20°C till used in DNA extraction. DNA was extracted as described by Waage *et al.* [16]. From each overnight culture, 100 ml of undiluted broth and 100 µl of broth was transferred to Eppendorf tubes and centrifuged at 14 900 g for 10 min in a microcentrifuge. The resulting pellets were resuspended in 50 µl PCR buffer with 1µl Proteinase K (0•2 mg ml^{-1}). After incubation at 37°C for 1 hour, the bacteria were lysed by boiling for 10 min. The samples were stored at -20°C overnight prior to PCR. After thawing at room temperature and centrifugation at 14 900 g for 5 min., 5 µl of supernatant was used in PCR as a template. The polymerase chain reaction was used to confirm the isolates of *Vibrio spp.* using the specific primers V.16S-700F 5□-CGGTGAAATGCG TAGAGAT-3□ andV.16S-1325R5□-TTACTAGCGATTCCGAGTTC-3□ [17]. PCR reaction contained 2 units of Ampli Taq Gold (Promega) and final concentrations of each deoxynucleoside triphosphate (dNTP) (Promega) and $MgCl_2$ of 0.2 mM and 1.5 mM, respectively and primer concentrations is 0.05 µM. Thus, a typical 20µl reaction mixture contained 1.5 µl DNA template, 0.2 µl of Ampli Taq Gold, 2 µl dNTPs stock (2 mM each), 2 µl 10x buffer,1.2 µl $MgCl_2$ stock (25 mM) and 1 µl of each 10 µM stock primer. The thermal cycling profile was as follows: a 15-min soak at 93°C followed by 35 cycles of 92°C for 40 s, 57°C for 1 min. and 72°C for 1.5 min. and a final soak at 72°C for 7 min. PCR experiments were carried out on an Applied Biosystems Gene Amp PCR system 9700 [17].

Detection of *Vibrio* spp. by Multiplex PCR: Multiplex PCR was used for the comprehensive detection of *V. vulnificus, V. parahaemolyticus* and *V. cholerae* according to Panicker *et al.* [18]. The targeted genes, oligonucleotide primer sets used for the detection of each of the three pathogens are listed in (Table 1). Each multiplex PCR amplification was performed with a 50µl reaction volume consisting of 10 ng of purified genomic DNA, 200 µM dNTPs (Sigma), 3 U of AmpliTaq DNA polymerase (Promega Corporation, Madison, Wis.) and PCR buffer F [60 mM Tris-Cl (pH 9.0), 15 mM $(NH_4)2SO_4$, 2 mM $MgCl_2$] or buffer C [50 mM Tris-Cl (pH 8.9), 50 mM KCl, 2.5 mM $MgCl_2$]. Amplification of the targeted genes for *V. cholerae* and *V. parahaemolyticus* was carried out with 1 µM each oligonucleotide primer. However, for the detection of *V. vulnificus*, 1 µM each of oligonucleotide primers F-vvh and R-vvh and 2 µM each of oligonucleotide primers F-viuB and R-viuB were used in order to achieve optimum multiplex PCR amplification of vvh and viuB, respectively. All PCR amplifications were performed by using a GeneAmp 2400 thermocycler (Perkin-Elmer, Shelton, Conn.) with parameters specific for the gene sets to be amplified. Reaction parameters included initial denaturation at 94°C for 3 min; 30 cycles of amplification with denaturation at 94°C for 1 min and primer annealing for 1 min at 55°C for *V. parahaemolyticus*, 65°C for *V. vulnificus*, or 60°C for *V. cholerae*; primer extension at 72°C for 1 min; and final extension of the incompletely synthesized DNA at 72°C for 5 min. Amplified products (5 µl) were analyzed by agarose gel electrophoresis (2% [wt/vol] Nusieve or 1% [wt/vol] SeaKem agarose [FMC Bioproducts, Rockland, Maine]). Gels were stained with ethidium bromide (0.005% [wt/vol]) and visualized under a Photoprep I UV transilluminator (Fotodyne, Inc., Hartland, Wis.).

Thereafter, the slides were neutralized with 0.4M. Tris pH 7.5 for 15 min. (3×), fixed in ethanol and dried. The slides were stained with 60 µl of 20µg/ml Ethidium bromide solution and viewed under a fluorescent microscope using a U-MNG filter (Olympus).

Table 1: Primer sets and target genes for *Vibrio* spp

Pathogen	Target Gene	Primer sequence	Amplicon Size, (kbp)
V. vulnificus	*Vvh*	F-VVH: 5□-TTCCAACTTCAAACCGAACTATGAC-3□	
		R-VVH: 5□-ATTCCAGTCGATGCGAATACGTTG-3□	0.205
	viuB	F-VIUB1728: 5□-GGTTGGGCACTAAAGGCAGATATA-3	
		R-VIUB2231: 5□- CGGCAGTGGACTAATACGCAGC-3□	0.504
V. parahaemolyticus	*Tlh*	F-TLH:5□-AAAGCGGATTATGCAGAAGCACTG-3□	
		R-TLH: 5□- GCTACTTTCTAGCATTTTCTCTGC-3□	0.45
	Tdh	F-TDH: 5□-GTAAAGGTCTCTGACTTTTGGAC-3□	
		R-TDH: 5□- TGGAATAGAACCTTCATCTTCACC-3□	0.269
	Trh	F-TRH: 5□-TTGGCTTCGATATTTTCAGTATCT-3□	
		R-TRH: 5□-CATAACAAACATATGCCCATTTCCG-3□	0.5
	ORF8	F-O3MM824: 5□-AGGACGCAGTTACGCTTGATG-3□	
		R-O3MM1192: 5□-CTAACGCATTGTCCCTTTGTAG-3□	0.369
V. cholerae	*omp*U	F-OMPU: 5□-ACGCTGACGGAATCAACCAAAG-3□	
		R-OMPU: 5□-GCGGAAGTTTGGCTTGAAGTAG-3□	0.869
	toxR	F-TOXR: 5□-CCTTCGATCCCCTAAGCAATAC-3□	
		R-TOXR: 5□-AGGGTTAGCAACGATGCGTAAG-3□	0.779
	tcpI	F-TCPI: 5□-TAGCCTTAGTTCTCAGCAGGCA-3□	
		R-TCPI: 5□-GGCAATAGTGTCGAGCTCGTTA-3□	0.862
Classical or El Tor	*hlyA*	F-HLYA: 5□-GGCAAACAGCGAAACAAATACC-3□	0.738
		R-HLYA: 5□-CTCAGCGGGCTAATACGGTTTA-3□	Or 0.727

RESULTS AND DISCUSSION

Sample collection and identification: Samples identification occurred in the laboratory and confirmed through the use of keys that were compared with correctly identified specimens in Department of Entomology, Faculty of Science, Ain Shams University. Aquatic insects and fish samples were identified as seen in Table (2).

Bacteriological examination:

Determination of total vibrios by MPN-TCBS method: The results of determination of total vibrios by MPN in water, aquatic insects and fish collected from River Nile at different polluted sites during one year (February 2009-January 2010) were represented in Tables (3-7). Table (3) showed the density of total vibrios detected in different polluted sites. It could be realized that the incidence of total vibrios was higher in warm months than cold ones. Water temperature has been shown to be a major factor influencing the occurrence and multiplication of *V. cholerae* in the aquatic environment [19]. Similar results were obtained by Shar *et al*. [20] who studied the seasonal distribution of two *Vibrio species*, *V. cholarae* and *V. mimicus*, in municipal water of three cities (Khairpur, sukkur and Rohri of Sindh, Pakistan. Results indicated higher incidence of the two pathogens in summer than winter. Monitoring of different

Table 2: Aquatic insects and fish species sampled from different polluted sites along River Nile, Egypt

Sampling site		Aquatic insects and fish spp.	Family	Order
The main stream of River Nile before branches	Aquatic insects	*Appasus urinator*	Belostomatidae	Hemiptera
		Enallagma vansomereni	Coenagriidae	Odonata
	Fish	*Oreochromis niloticus*		
		(Nile Tilapia: 13-16 cm, 55-64 g)	Cichlidae	Perciformes
The mixed point of agriculture drain	Aquatic insects	*Appasus urinator*	Belostomatidae	Hemiptera
		Hydaticus leander	Dytiscidae	Coleoptera
	Fish	*Oreochromis niloticus*		
		(Nile Tilapia: 13-16 cm, 55-64 g)	Cichlidae	Perciformes
The mixed point of wastewater	Aquatic insects	*Eristalis* spp. (rat-tailed maggot)	Syrphidae	Diptera
		Stratiomys spp.	Stratiomyidae	Diptera
	Fish	*Silurus triostegus*		
		(Cat fish: 44-57 cm, 500-1000 g)	Siluridae	Siluriformes

Table 3: Determination of total vibrios in water by most probable number technique (MPN)/100ml collected from River Nile at different polluted sites

	Main stream of River Nile before branches			Mixed point of agriculture drain			Mixed point of wastewater		
Month	*V. cholerae*	Other vibrios	Total vibrios	*V. cholerae*	Other vibrios	Total vibrios	*V. cholerae*	Other vibrios	Total vibrios
February 2009	ND	ND	ND	1.5×10^2	1.1×10^2	2.6×10^2	2.1×10^3	ND	2.1×10^3
March	ND	ND	ND	ND	ND	ND	1.1×10^2	ND	1.1×10^2
April	ND	ND	ND	1.5×10^2	1.1×10^2	2.6×10^2	2.1×10^3	ND	2.1×10^3
May	ND	ND	ND	ND	ND	ND	ND	9.3×10^3	9.3×10^3
June	ND	ND	ND	2.3×10^2	ND	2.3×10^2	2.1×10^3	ND	2.1×10^3
July	ND	ND	ND	ND	ND	ND	ND	1.1×10^3	1.1×10^3
August	ND	ND	ND	ND	ND	ND	ND	ND	ND
September	ND	ND	ND	1.1×10^4	ND	1.1×10^4	2.4×10^3	2.4×10^3	4.4×10^3
October	ND	ND	ND	4.6×10^4	2.4×10^3	4.8×10^4	ND	ND	ND
November	ND	ND	ND	1.1×10^4	ND	1.1×10^4	ND	ND	ND
December	ND	ND	ND	ND	7.0×10^2	7.0×10^2	2.4×10^2	2.1×10^2	4.5×10^2
January 2010	ND	ND	ND	ND	ND	ND	ND	ND	ND
Minimum	ND	ND	ND	1.5×10^2	7.0×10^2	7.0×10^2	1.1×10^2	2.1×10^2	1.1×10^2
Maximum	ND	ND	ND	4.6×10^4	2.4×10^3	4.8×10^4	2.4×10^3	9.3×10^3	9.3×10^3
Average	ND	ND	ND	1.1×10^4	8.3×10^2	1.0×10^4	1.5×10^3	3.2×10^3	2.7×10^3

(ND: Not detected)

Table 4: Determination of total vibrios by most probable number technique (MPN)/g in different aquatic insects collected from River Nile at different polluted sites

	Main stream of River Nile before branches			Mixed point of agriculture drain			Mixed point of wastewater		
Month	*V. cholerae*	Other vibrios	Total vibrios	*V. cholerae*	Other vibrios	Total vibrios	*V. cholerae*	Other vibrios	Total vibrios
February 2009	ND	ND	ND	2.8×10^3	1.1×10^5	1.1×10^5	7.5×10^3	1.1×10^5	1.1×10^5
March	ND	ND	ND	ND	2.4×10^4	2.4×10^4	ND	2.3×10^3	2.3×10^3
April	ND	ND	ND	ND	1.1×10^5	1.1×10^5	1.1×10^3	2.4×10^4	2.5×10^4
May	ND	ND	ND	ND	ND	ND	2.3×10^3	1.1×10^3	3.4×10^3
June	ND	ND	ND	ND	2.1×10^4	2.1×10^4	7.5×10^3	1.1×10^4	1.8×10^4
July	ND	ND	ND	ND	ND	ND	2.1×10^4	2.1×10^3	2.3×10^4
August	ND	ND	ND	ND	ND	ND	1.1×10^3	9.3×10^5	9.3×10^5
September	ND	1.1×10^1	1.1×10^1	1.1×10^6	ND	1.1×10^6	1.1×10^5	2.1×10^5	3.2×10^5
October	ND	ND	ND	1.5×10^3	ND	1.5×10^3	ND	ND	ND
November	4.6×10^1	2.8×10^3	2.8×10^3	1.1×10^4	ND	1.1×10^4	1.1×10^3	1.1×10^4	1.2×10^4
December	ND	ND	ND	1.1×10^3	ND	1.1×10^3	2.0×10^3	2.1×10^3	4.1×10^3
January 2010	ND	ND	ND	ND	ND	ND	1.1×10^2	1.1×10^3	1.2×10^3
Minimum	4.6×10^1	1.1×10^1	1.1×10^1	1.5×10^3	2.1×10^4	1.1×10^3	1.1×10^2	1.1×10^3	1.2×10^3
Maximum	4.6×10^1	2.8×10^3	2.8×10^3	1.1×10^6	2.4×10^4	1.1×10^6	1.1×10^5	9.3×10^5	9.3×10^5

(ND: Not detected)

Table 5: Determination of total vibrios by most probable number technique (MPN)/g in fish collected from the main stream of River Nile before branches during one year

	Fish organs								
	Muscle			Liver			Gills		
Month	*V. cholerae*	Other vibrios	Total vibrios	*V. cholerae*	Other vibrios	Total vibrios	*V. cholerae*	Other vibrios	Total vibrios
February 2009	ND	ND	ND	ND	ND	ND	ND	ND	ND
March	ND	ND	ND	ND	ND	ND	ND	ND	ND
April	ND	ND	ND	ND	ND	ND	ND	ND	ND
May	ND	ND	ND	ND	ND	ND	ND	ND	ND
June	ND	ND	ND	ND	ND	ND	7.0×10^2	ND	7.0×10^2
July	ND	ND	ND	ND	ND	ND	ND	ND	ND
August	ND	ND	ND	ND	ND	ND	9.3×10^3	ND	9.3×10^3
September	ND	ND	ND	ND	ND	ND	7.0×10^2	9.0×10^2	1.6×10^3
October	ND	ND	ND	ND	ND	ND	ND	ND	ND
November	ND	ND	ND	ND	ND	ND	ND	ND	ND
December	ND	ND	ND	ND	ND	ND	ND	ND	ND
January 2010	ND	ND	ND	ND	ND	ND	ND	ND	ND
Minimum	ND	ND	ND	ND	ND	ND	7.0×10^2	9.0×10^2	7.0×10^2
Maximum	ND	ND	ND	ND	ND	ND	9.3×10^3	9.0×10^2	9.3×10^3
Average	ND	ND	ND	ND	ND	ND	3.5×10^3	9.0×10^2	3.8×10^3

Table 6: Determination of total vibrios by most probable number technique (MPN)/g in fish collected from mixed point of agriculture drain during one year

	Fish organs								
	Muscle			Liver			Gills		
Month	*V. cholerae*	Other vibrios	Total vibrios	*V. cholerae*	Other vibrios	Total vibrios	*V. cholerae*	Other vibrios	Total vibrios
February 2009	ND	ND	ND	ND	ND	ND	2.1×10^3	ND	2.1×10^3
March	ND	ND	ND	ND	ND	ND	2.1×10^3	ND	2.1×10^3
April	ND	ND	ND	ND	ND	ND	ND	ND	ND
May	ND	ND	ND	3.9×10^3	4.6×10^4	4.9×10^4	ND	9.3×10^3	9.3×10^3
June	ND	ND	ND	ND	ND	ND	ND	ND	ND
July	ND	ND	ND	ND	ND	ND	ND	ND	ND
August	ND	ND	ND	ND	ND	ND	4.6×10^4	ND	4.6×10^4
September	ND	ND	ND	ND	9.3×10^3	9.3×10^3	1.1×10^4	9.0×10^2	1.1×10^4
October	ND	ND	ND	ND	ND	ND	2.8×10^3	ND	2.8×10^3
November	ND	ND	ND	ND	ND	ND	9.3×10^3	ND	9.3×10^3
December	ND	ND	ND	ND	ND	ND	7.5×10^3	ND	7.5×10^3
January 2010	ND	ND	ND	ND	ND	ND	2.8×10^3	ND	2.8×10^3
Minimum	ND	ND	ND	3.9×10^3	9.3×10^3	9.3×10^3	2.1×10^3	9.0×10^2	2.1×10^3
Maximum	ND	ND	ND	3.9×10^3	4.6×10^4	4.9×10^4	4.6×10^4	9.3×10^3	4.6×10^4
Average	ND	ND	ND	3.9×10^3	2.7×10^4	2.9×10^4	1.0×10^4	5.1×10^3	1.0×10^4

(ND: Not detected)

Table 7: Determination of total vibrios by most probable number technique (MPN) / gm in fish collected from mixed point of wastewater during one year

	Fish organs								
	Muscle			Liver			Gills		
Month	*V. cholerae*	Other vibrios	Total vibrios	*V. cholerae*	Other vibrios	Total vibrios	*V. cholerae*	Other vibrios	Total vibrios
February 2009	ND	ND	ND	ND	ND	ND	ND	ND	ND
March	ND	ND	ND	ND	ND	ND	4.8×10^4	ND	4.8×10^4
April	ND	ND	ND	ND	ND	ND	ND	ND	ND
May	ND	ND	ND	ND	3.9×10^3	3.9×10^3	7.0×10^2	2.3×10^3	3.0×10^3
June	ND	ND	ND	6.4×10^3	2.1×10^4	2.7×10^4	ND	2.1×10^3	2.1×10^3
July	ND	ND	ND	ND	3.9×10^3	3.9×10^3	7.0×10^2	2.3×10^3	3.0×10^3
August	ND	ND	ND	9.0×10^2	ND	9.0×10^2	4.6×10^4	ND	4.6×10^4
September	ND	ND	ND	7.0×10^2	ND	9.0×10^2	ND	ND	ND
October	ND	ND	ND	ND	ND	ND	ND	ND	ND
November	ND	ND	ND	ND	ND	ND	4.8×10^4	ND	4.8×10^4
December	ND	ND	ND	9.0×10^2	ND	9.0×10^2	ND	ND	ND
January 2010	ND	ND	ND	ND	ND	ND	ND	ND	ND
Minimum	ND	ND	ND	7.0×10^2	3.9×10^3	9.0×10^2	7.0×10^2	2.1×10^3	2.1×10^3
Maximum	ND	ND	ND	6.4×10^3	2.1×10^4	2.7×10^4	4.8×10^4	2.3×10^3	4.8×10^4
Average	ND	ND	ND	2.2×10^3	9.6×10^3	6.2×10^3	2.8×10^4	2.2×10^3	2.5×10^4

(ND: Not detected)

pathogens in water could be used as a tool to assess the health status of the community [21]. Kamel *et al.* [22] studied the microbiological profile of El-Salam canal (River Nile mixed with agriculture drain) and El-Adlia site (River Nile, Damietta branch) during one year. The results revealed that the density of total vibrios at El-Adlia site fluctuated from 90 to 5.3×10^4 CFU/100ml, whereas the density along El-Salam canal ranged between 1.2×10^5 and 20 CFU/100ml as a mean value. So, total vibrios were slightly high along El-Salam canal because the condition of the water is suitable to encouraging growth of these pathogens. In addition, the biochemical identification of isolates has demonstrated the predominance of *V. cholera* and *Listeria* (*L.*) *monocytogenes* over the remaining species [23]. Salyers and Whitt [24] concluded that, *V. cholera* species can grow in both salt water and fresh water. The total presumptive and culturable *Vibrio* counts (i.e., growing on TCBS) in west coast water samples with a mean values ranged from $4.73 \pm 4.69 \times 10^4$ CFU/ml to $5.48 \pm 3.43 \times 10^2$ CFU/ml in water samples collected from the east and west coast of India [25]. Determination of total vibrios by MPN/g in aquatic insects collected from River Nile at different polluted sites during one year (February 2009-January 2010) was represented in Table (4). It could be concluded that aquatic insects may harbor total vibrios in freshwater of different

contamination levels. *Vibrio* spp. were isolated from many insects in recent studies [26]. It was found that various *V. cholerae* non-O1 and non-O139 serogroups were isolated from chironomid egg masses from different freshwater bodies in Israel, India and Africa that supported the hypothesis that the association found between chironomids and the cholera bacteria was not a rare coincidence [6]. The utility of *Drosophila melanogaster* as a model host for human pathogens is well-established [27]. Also total vibrios were determined in fish collected from River Nile from different pollution sites in 3 organs; muscle, liver and gills (Tables 5-7). It could be realized that fish muscles did not contain any *Vibrio* spp. while in fish liver total vibrios were detected only in two months (May and September) in fish collected from the mixed point of agriculture drain and not detected in the main stream of River Nile before branches. Concerning fish gills; it was found that total vibrios were detected in gills all over the year except 3 months in fish collected from the mixed point of agriculture drain. In the natural environment, *V. cholerae* is closely associated with arthropods [27] and many have suggested that insects serve as vectors [28] or reservoirs [29] of *V. cholerae*. Halpern *et al*. [30] suggested that chironomid egg masses are a natural reservoir for *Aeromonas* species as well as *V. cholera*. Temperature has been found to be a major factor in both the seasonal and geographical Distribution of *V. parahaemolyticus* in shellfish-growing areas of the temperate region [31].

DePaola *et al*. [32] noted that in Alabama oysters, the *V. parahaemolyticus* levels were <100 CFU/g during December to March. Many studies revealed that insects may aid in pathogens distribution through water especially that fish live in water and feed on insects as tilapia fish is omnivorous and feed on many types of feed. Senderovich *et al*. [26] suggested that fish are reservoirs of *V. cholerae*. Without PCR confirmation, the MPN results were misleading. This is because that the medium APW used for MPN tube cultures is not highly selective and thus could allow other *Vibrio* species and aquatic microorganisms to proliferate.

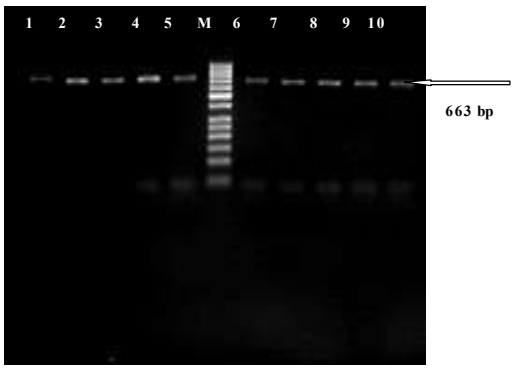

Fig. 1: Detection of *Vibrio* spp. by PCR in water (lanes: 2-4), aquatic insects (lanes: 5-7) and fish (lanes: 8-10) collected from River Nile at different pollution sites.
Lane (1): +v control, specific band: 663 bp,
Marker:
50,100,150,200,250,300,400,500,600,700,800,900,1000 bp

Determination of *Vibrio* spp. by PCR and Multiplex PCR: The results in (Fig. 1) and Table (8) showed the incidence of total vibrios in water, aquatic insects and fish collected

Table 8: The positive determination rate of total vibrios in different samples

				No. of +ve *Vibrio* spp. tested by PCR	
Sample	Area description		No. of isolates tested	No.	%
Water	Main stream of River Nile before branches	0	0	0	
	Mixed point of wastewater	50	23	46	
	Mixed point of agriculture drain	50	19	38	
Aquatic insects	Main stream of River Nile before branches	15	4	27	
	Mixed point of wastewater	105	63	60	
	Mixed point of agriculture drain	45	31	69	
Fish	Main stream of River Nile before branches	Muscle	0	0	0
		Liver	0	0	0
		Gills	20	8	40
	Mixed point of wastewater	Muscles	0	0	0
		Liver	35	14	40
		Gills	40	26	65
	Mixed point of agriculture drain	Muscle	0	0	0
		Liver	15	3	20
		Gills	50	19	38
Total isolates			425	202	

from River Nile at different pollution sites. Samples were detected by MPN technique and confirmed by PCR techniques [12]. The PCR assay provides a simple, rapid and reliable tool for identification of the major *Vibrio* pathogens in different samples. The PCR was used to confirm the identities of *Vibrio species* using the specific primers amplifying the 16S rRNA fragment [17]. Modern molecular biology methods have more and more been used in pathogenic bacteria detecting due to their good specificity and high sensitivity. The PCR procedures are clearly rapid and highly specific for determination of *V. parahaemolyticus* [12, 33]. The MPN-PCR method was used recently to enumerate *V. parahaemolyticus* in environmental and food samples. A MPN method combined with a PCR procedure for the detection of a specific gene of an organism is more valuable than isolation of the organism itself [34]. Sensitive and rapid molecular methods such as PCR have been applied to identify the presence of tdh and trh genes from *V. parahaemolyticus* [35, 36]. PCR and other genotypic assays have been applied to environmental studies [37, 38] and seafood surveys [36, 37]. Multiplex PCR was used for the comprehensive detection of *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae*. Multiplex PCR was used with *vvh* and *viuB* for *Vibrio vulnificus*, with *ompU*, *toxR*, *tcpI* and *hlyA* for *V. cholerae* and with *tlh*, *tdh*, *trh* and open reading frame 8 for *V. parahaemolyticus* helped to ensure that total and pathogenic strains, including subtypes of the three *Vibrio* spp., could be detected and discriminated according to Panicker *et al*. [18]. Many PCR methods have been developed for the identification of bacterial pathogens in aquacultures [39]. Although many of these protocols are based on the amplification of 16S and 23S rRNA genes [40, 41], which are found in all eubacteria, there is a high degree of genetic similarity for these genes across taxa; therefore, the specificity of the detection method can be compromised [42]. Alternatively, bacterium-specific genes (e.g., virulence loci) can be used as targets for PCR amplification to permit more specific detection [43] as well as subspecies and strain differentiation [44, 45].

Conventional PCR is used to amplify a single gene target, whereas multiplex PCR involves amplifying multiple gene products in a single reaction; the latter method has been used successfully to detect fish pathogens [45]. Results of the present study using multiplex PCR for detection of most 3 important *Vibrio* spp.; *V. vulnificus*, *V. parahaemolyticus* and *V. cholera* indicated -ve *V.* when tested by PCR for tdh gene. The two isolates were obtained from aquatic insects from the main stream of River Nile before branches and from water collected from the mixed point of wastewater. Similar results were obtained by Luan *et al*. [12] who found that only one sample from shrimp liver and pancreas was positive for tdh gene possessing cells by the direct PCR method. *V. parahaemolyticus* is an important human pathogen which can cause gastroenteritis when consumed in raw or partially-cooked seafood. A multiplex PCR was used to detect virulence genes of *V. parahaemolyticus*. Results indicated that only one strain showed positive amplification for tdh gen. Similar results were obtained by Lee and Pan [46]. Most strains of *V. parahaemolyticus* associated with human disease produce tdh or/and trh [36, 47] and the identification of *V. parahaemolyticus* by PCR targeting the tdh gene has been reported [46, 48]. The multiplex PCR approach was successfully used to detect various strains of *V. parahaemolyticus* in seeded oyster tissue homogenate. This high level of sensitivity of detection of this pathogen within 8 hours of preenrichment is well within the action level (10^4 cfu per 1 g of shell stock). Compared to conventional microbiological culture methods, this multiplex PCR approach is rapid and reliable for accomplishing a comprehensive detection of *V. parahaemolyticus* in shellfish [49].

It could be concluded that special attention should be given to water pollution as this affects fish and so becoming danger to human and animal health. Also, improving wastewater treatment and monitoring the hygienic quality of River Nile is a must to control the outbreaks of waterborne diseases.

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