Phenotypic and Genotypic characterization of Vibrio species isolated from marine fishes

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A B S T R A C T

Vibriosis is considered the most important threatening disease problem facing aqua culture. The bacteria occur widely in aquatic environment and are part of the normal flora of coastal seawater and are opportunistic pathogens in marine animals. A total number of (311) fish, *(97) Seabream*, *(118) Seabass*, (96) *Mugil capito* were collected from Kafr Hamedo and Ezzbet El Borg marine water fish farm (Damietta governorate), El Manzala and Mansoura Fish Market (EL-Dakahlia governorate) in the period from March 2016 to April 2017 during the four seasons. The clinical examination of diseased fish revealed that, presence of redness at base of anal fin and erosion of caudal fin, presence of ulcers in skin, darkness of the skin, detached scales, fin erosion, corneal opacity and pale gills. Post mortem examination of infected fishes revealed that, liver appeared enlarged, congested or pale with engorged gall bladder, splenomegaly, congested kidney and hemorrhage in abdominal cavity. Congested gills with excessive amount of mucous and enlarged liver with hemorrhagic patches on its edges. The isolated bacteria on thiosulfate citrate bile salt sucrose agar (TCBS) gave yellow colonies for *Vibrio alginolyticus* and gave green colonies for *Vibrio parahemolyticus*. The strains were tested according to their susceptibility as resistant, intermediate or sensitive for each antibiotic group. *Vibrio alginolyticus and Vibrio parahemolyticus* revealed sensitivity to ciprofloxacin (Cipro5µg) and novobiocine (NV30µg). Moreover intermediate sensitivity was found to oxytetracycline (OT 30 µg). Resistance was observed to amoxicillin (Aml 10 µg) and cholistine sulphate (CT 10 µg). The isolated bacteria (Vibrio alginolyticus and Vibrio parahemolyticus) were 171/311 (54.98%) from infected fish where the total number of Seabass 72/118 (61%), Seabream 47/97 (48.45%) and Mugil capito 52/96 (54.17%). The highest infection rate was recorded by summer (100% , 82.61% , 80%), winter (73% , 58.33% , 54.54%) , spring (56% , 42.11% , 69.23%) and autumn (36% , 30.23% , 40.38%) for Seabass, Seabream and Mugil capito respectively. PCR yielded a single specific and clear amplified band of expected size 737 bp for collagenase gene and 387 bp for pR72H gene for *V. alginolyticus* and *V. parahemolyticus* respectively.

Key words: *V. alginolyticus, V. parahemolyticus,* Marine fishes, Collagenase and pR72H genes.

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1. INTRODUCTION

Egypt's aquaculture production were estimated over 705,490 tons in 2009 as the largest of any African country and 11th in the global production and the importance of this sector as it is providing a cheap source of protein for the Egyptian people (Macfadyen et al.,2011; Sadek, 2013). Fisheries represent an important sector in the Egyptian national income structure. In the fisheries economy, marine fishes represent the major investment choices for the national fishermen. Marine fishes are liable to variable number of environmental stressors, including chemical, natural and biological invaders. Such stressors are the main predisposing factors for the chronic immunosuppression of marine aquatic animals in the affected marine habitat. As an ultimate fate for the staggering immuno-suppression of fishes, bacterial invasion will be the most probable event (Ellis, 1999). Further, the bacterial invasion of any marine species could possibly exacerbate under the triggering effect of the fluctuating climatic changes (Wedemeyer, 1996). Outbreaks of bacterial diseases are largely responsible for the high mortality of wild and farm-cultured fish causes severe economic losses to fish farms (Olsson et al., 1998). Recently, vibriosis as an important pathogenic cause for outbreaks in Egyptian aquaculture industry

was recorded in many studies (Abd El-Galil and Mohamed, 2012; Abd-Elghany and Sallam, 2013; Abdel-Aziz et al., 2013; Eissa et al., 2013 ; El-Hady et al.,2015). Vibriosis is a human illness caused by pathogenic species of the family Vibrionaceae (CDC 2016). The genus Vibrio comprises more than 45 species, most of which are widely distributed in marine environment. The Vibrio species affected all type of fish of either marine or fresh water fish all over the world in different areas of Asia, America, Australia, Africa and Europe (Reham, 2009). *Vibrio alginolyticus* and *Vibrio parahemolyticus* are responsible for mass mortalities among fish stocks in many marine fish farms throughout the Mediterranean area and severe economic losses in aquaculture worldwide (Snoussi et al., 2008). According to Marhual et al., (2010) and Letchumanan et al.,(2015). *Vibrio alginolyticus* and *Vibrio parahaemolyticus* are important halophilic Gram negative pathogens, non-spore forming, curved rod shaped bacterium which cause serious episode to marine fish and shellfish and also naturally lives in marine environments worldwide. Gastroenteritis caused by *V. parahaemolyticus* is mainly characterized by reddish watery bloody diarrhea "Meat Washed", abdominal cramps, nausea, vomiting, headache and low grade fever (Honda and lida., 1993). Isolation and identification of *Vibrio* can be made by using thio sulphate citrate bile salt sucrose agar (TCBS) which is the primary plating medium universally used for the selective isolation of vibrios such as *Vibrio alginolyticus* and *V. parahaemolyticus* from a variety of clinical and nonclinical specimens. The later produces non-sucrose fermenting typical round (2-3 mm in diameter), green or blue center colonies on TCBS Agar *(*Elliot et al., 1992*).*

This study aimed to conduct phenotypic and genotypic identification of *Vibrios* isolated from marine fishes and to evaluate the seasonal prevalence of bacterial isolates among the examined fishes.

2. MATERIALS AND METHODS

2.1. Sampling

A total number of (311) fish, *(97) Seabream*, *(118) Seabass,* (96) *Mugil capito,* with body weight ranging from (70 – 450 g) were freshly collected at random samples from Kafr Hamedo and Ezzbet El Borg marine water fish farm (Damitta governorate) , El Manzala and Mansoura Fish Market (EL-Dakahlia governorate) in the period from March 2016 to April 2017 during the four seasons. Fishes were transferred alive in plastic tank with air blower, freshly dead samples were kept in ice boxes and were transported to Animal Health Research Institute (AHRI) - Al Mansoura branch and subjected to clinical, post mortem and bacteriological examination for isolation of *Vibrio* spp.

2.2. Clinical examination

External clinical examination was performed using the method described by Schaperclaus et al. (1992) and internal (Post mortem) examination according to Austin and Austin (2007).

2.3. Bacteriological examination

Sampling and primary isolation of bacteria was carried out under complete aseptic conditions then inoculated into tryptic soya broth with 2% NaCl and incubated at 25^{o}C for 24hr. A loopful of incubated broth streaked on thiosulfate citrate bile salt sucrose agar (TCBS) and incubated at 25^{o}C for 24hr. according to *(Noga, 1996),* Purification of bacterial isolates *(Austin and Austin, 2012),* Identification of bacterial isolates through Gram stain (Shape and arrangement of bacteria) according to method described by Lucky (1977), Motility test and Oxidase test (Buller, 2004).

Table 1: Target gene, oligonucleotide primer sequence and PCR amplicon (bp) for *Vibrio alginolyticus* and *Vibrio parahemolyticus*

Gene	Sequence	Molecular weight	Reference
Collagenase	F: (5-CGAGTACAGTCACTTGAAAGCC-3) R: (5- CACAACAGAACTCGCGTTACC-3)	737 Bp	Di pino et al.,2005
PR72H	Vp32:(5-CGAATCCTTGAACATACGCAGC-3) VP33: (5-TGCGAATTCGATAGGGTGTTAACC-3)	387 Bp	Lee et al.,1995

Identification of the isolates by API® 20 E: Analytical profile index system according manufacture guide (BioMerieux, Paris, France), Growth of bacteria in different concentration of sodium chloride, other conventional test: Catalase test, V.P. reaction and Hydrogen sulphide on TSI media.

2.4. Molecular identification of Vibrio by polymerase chain reaction (PCR)

DNA molecular marker 50 to 2000 bp ladder (Sigma) and procedures according to manufacturer protocol of Omega Co. (USA. LMt.)

Procedures for total genomic of *Vibrio sp.* Samples were done according to protocol of Omega Co. (USA. LMt.) The reaction consists of 40 cycles; each cycle consisted of denaturation at 94 ^{0}C for 30 sec followed by annealing at 30 ^{0}C for 30 sec and extension at 72 ^{0}C for 30 sec (Di pino et al., 2005). There was an initial delay for 15 min at 95 ^{0}C at the beginning of the first cycle and 10 min delay at 72^{0}C at the end of the last cycle as a post extension step the product was stored at –20 C or 4 ^{0}C. Gel documentation system was applied for data analysis using Total lab analysis software (Geldoc-it, UVP, England)

2.5. Antibiogram test

Antibiotic Sensitivity Discs (oxoid) as Oxytetracycline (OT 30ug), Ciprofloxacine (Cipro5ug), Cholisitine Sulphate (CT 10ug), Amoxyciline (Aml 10ug) and Novobiocine (NV 30ug) for differentiation between vibrio spp. and other Gram negative bacterial isolates .

The disc diffusion method described by Koneman et al., (1992) and Quinn et al., (2002). Commercially available antibacterial disks (Oxoid) was dispensed on the surface of the medium with sterile forceps and incubated for 24 h at 25°C. After incubation of the plates, the degree of sensitivity was determined by measuring the zone of inhibition around each disk which produced by diffusion of antimicrobial agents from the discs into surrounding medium.

3. RESULTS

3.1. Diagnosis of Vibriosis

3.1.1. Clinical signs and post mortem changes of infected fishes with Vibriosis

Result of clinical examination of diseased fish revealed that, presence of redness at base of anal fin and erosion of caudal fin. Presence of ulcers in skin, darkness of the skin, detached scales, fin erosion, hemorrhages on several parts of the body surface, Hemorrhagic areas around the mouth, corneal opacity and pale gills. The post mortem examination of infected fishes revealed that, liver appeared enlarged, congested or pale with engorged gall bladder, splenomegaly, congested kidney and hemorrhage in abdominal cavity.

3.1.2. Biochemical identification

Vibrio spp. is Oxidase positive and catalase positive, sensitive to Novobiocine (30 µg) and Vibriostatic disc O/129 (150 µg), grow at wide range of temperature (20-35^{o}C) and salinity (2- 8 % NaCl).

Figure (1): Agarose gel electrophoresis of products obtained after PCR amplification of collagenase gene of three Vibrio alginolyticus strains extracted from Mugil capito yielded (737 Bp) using forward and reverse primers. Ld. Indicate 100bp size ladder,1 indicate Vibrio alginolyticus strains isolated from spleen.2 indicate Vibrio alginolyticus strains isolated from liver.3 indicate Vibrio alginolyticus strains isolated from kidney

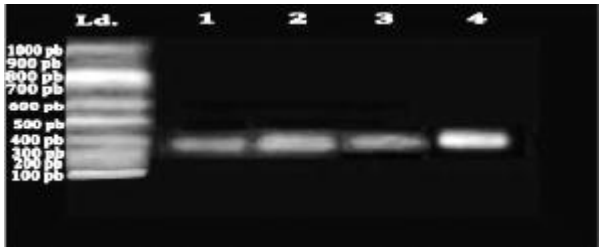

Figure (2): Agarose gel electrophoresis of products obtained after PCR amplification of pR72H gene of four Vibrio parahemolyticus strains extracted from Mugil capito yielded (387Bp) using forward and reverse primers. Ld. Indicates 100bp size ladder, 1 indicates Vibrio alginolyticus strains isolated from gill. 2 indicate Vibrio alginolyticus strains isolated from spleen.3 indicate Vibrio alginolyticus strains isolated from liver.4 indicate Vibrio alginolyticus strains isolated from kidney.

It was Positive reaction for arginine decarboxylase, indole production, vogus-proskauer tryptophan desaminase, gelatinase, glucose and mannitol fermentation. , Negative reaction for H2S production, urea hydrolysis, Ortho-nitro phenylgalactosidase, Lysine and Ornithine decarboxylase, citrate utilization, inositol, sorbitol, mellibiose, and arabinose fermentation. No growth appeared at 0%, NaCl but positive at 2%, 4% 6%, 8% NaCl as shown in table (2).

3.1.3. Antimicrobial susceptibility testing

The strains were tested according to their susceptibility as resistant, intermediate or sensitive for each antibiotic group. *Vibrio alginolyticus and Vibrio parahemolyticus* revealed sensitivity to ciprofloxacin (Cipro5µg), novobiocine (NV30µg). Moreover intermediate sensitivity was found to oxytetracycline (OT 30 µg). While resistance was observed to amoxicillin (Aml 10 µg) and cholistine sulphate (CT 10 µg) as illustrated in table (3).

3.1.4. Molecular identification

The amplified patterns obtained by PCR with tested *V. alginolyticus and V. parahemolyticus* strains. All isolates were positively reacted to the collagenase gene primers. Each strain gave almost a common band with the same molecular weight observed in the different strains. The three isolates of *V. alginolyticus* yielded a single band of amplified product at (737 bp) corresponding to collagenase gene as shown in figure (1) .Also The four isolates of *V. parahemolyticus* yielded a single band of amplified product at (387 bp)corresponding to pR72H gene as shown in figure (2).

3.2. Prevalence of Vibrio spp. Infection

This study showed some epidemiological characteristics of *Vibriosis* in Sea bass, Sea bream and Mugil capito represented in the etiological agent and its relationship with its prevalence in fish and in between seasons.

As shown in (table 4), which showed seasonal prevalence of *Vibrio spp.* infection in Sea bass, Sea bream and Mugil capito. Total 311 fishes (118 Sea bass, 97 Sea bream, 96 Mugil capito) were examined microbiologically for investigating the seasonal occurrence of *Vibrio spp.* infection. The total number of infected fish was 171 with 54.98 % prevalence rate. For Sea bass, the total number of infected fish was 72 from 118 with 61 % prevalence rate, the highest infection rate was recorded during summer season with prevalence rate 100% followed by winter 73 %, spring 56% and the lowest was autumn with prevalence rate 36%.

For Sea bream, the total number of infected fish was 47 from 97 with 48.45 % prevalence rate, the highest infection rate was recorded during summer season with prevalence rate 82.61 % followed by winter 58.33 %, spring 42.11 % and the lowest was autumn with prevalence rate 30.23 %. For Mugil capito, the total number of infected fish was 52 from 96 with 54.17 % prevalence rate, the highest infection rate was recorded during summer season with prevalence rate 80 % followed by spring 69.23 %, winter 54.54 % and the lowest was autumn with prevalence rate 40.38 %. Throughout the study two main *Vibrio spp.* were isolated and identified as *Vibrio alginolyticus* and *Vibrio parahemolyticus* infection. As shown in (table 5), the occurrence of *V. alginolyticus* and *V. parahemolyticus* infections in Sea bass in examined organs was , the liver was the highest infected organ with *V. alginolyticus* 39.13 % followed by spleen 37% and kidney 20 % and gills 4.35 % . Also, the liver was the highest infected organ with *V. parahemolyticus* 42.3 % followed by spleen 31% and kidney 15.4 % and gills 11.54 %. The occurrence of *V. alginolyticus* and *V. parahemolyticus* infections in Sea bream in examined organs was , the liver was the highest infected organ with *V. alginolyticus* 35.3 % followed by kidney 29.4% and spleen 20.59 % and gills 14.71 % . Also, the liver was the highest infected organ with *V. parahemolyticus* 38.5 % followed by kidney 30.8% and spleen 23.1 % and gills 7.7 %. The occurrence of *V. alginolyticus* and *V. parahemolyticus* infections in Mugil capito in examined organs was , the liver was the highest infected organ with *V. alginolyticus* 33.33 % followed by kidney 30.30% and spleen 24.2 % and gills 12.1 % . Also, kidney was the highest infected organ with *V. parahemolyticus* 36.8 % followed by liver 31.6% and spleen 21 % and gills 10.5%.

Table 2: Growth of Vibrio species on peptone water supplemented with different concentration of sodium chloride

Different conc. of salt (NaCl)	Vibrio Parahemolyticus	Vibrio alginolyticus
Peptone water +0% NaCl	-	-
Peptone water +2% NaCl	+	+
Peptone water +4% NaCl	+	+
Peptone water +6% NaCl	+	+
Peptone water +8% NaCl	+	+

- Indicate no turbidity of peptone water (negative result), + Indicate turbidity of peptone water (positive result).

Table 3: Showing antimicrobial resistance patterns of Vibrio strains

Antimicrobial agents	Symbol	Disk potency (µg)	*Vibrio alginolyticus*		*Vibrio parahemolyticus*	
			Inhibition zone (mm)	Interpretation	Inhibition zone (mm)	Interpretation
Oxytetracycline	OT	30 µg	18	Intermediate	13	Intermediate
Ciprofloxacin	Cipro	5 µg	19	Sensitive	17	Sensitive
Amoxicillin	Aml	10µg	-	Resistant	-	Resistant
Colstin sulphate	CT	10µg	-	Resistant	-	Resistant
Novobiocine	NV	30 µg	17	Sensitive	15	Sensitive

Table (4): Prevalence of bacteria in different types of fishes in relation to season

Fish	Total number of infected fish / Total number of examined fish				Total
	winter	spring	summer	autumn	
Seabass	11 / 15 (73.3%)	13 / 23 (56%)	30 / 30 (100%)	18 / 50 (36%)	72 / 118 (61%)
Seabream	7 / 12 (58.33%)	8 / 19 (42.11%)	19 / 23 (82.61%)	13 / 43 (30.23%)	47 / 97 (48.45%)
Mugil capito	6 / 11 (54.54%)	9 / 13 (69.23%)	16 / 20 (80%)	21 / 52 (40.38%)	52 / 96 (54.17%)

Table (5): Prevalence of Vibrio species isolated from different organs of naturally infected fishes

Isolated bacteria	Fish		Liver		Gills		Kidneys		Spleen	
		Total isolate	No.	%	No.	%	No.	%	No.	%
V.alginolyticus	Seabass	46	18	39.13%	2	4.35%	9	20%	17	37%
V.parahemolyticus		26	11	42.3%	3	11.54%	4	15.4%	8	31%
V.alginolyticus	Seabream	34	12	35.3%	5	14.71%	10	29.4%	7	20.59%
V.parahemolyticus		13	5	38.5%	1	7.7%	4	30.8%	3	23.1%
V.alginolyticus	Mugil capito	33	11	33.33%	4	12.1%	10	30.30%	8	24.2%
V.parahemolyticus		19	6	31.6%	2	10.5%	7	36.8%	4	21%

4. DISCUSSION

The results of the clinical signs examination of the infected Seabass , Seabream and Mugil Capito revealed the presence of hemorrhages at base of anal fin , erosion of caudal fin, darkness of the skin, presence of ulcers , detached scales, hemorrhages on several parts of the body surface, Hemorrhagic areas around the mouth, corneal opacity and pale gills, this is in agreement with Fryer et al.;(1972) and Levin et al.;(1972). While the post mortem examination of the infected fishes revealed that, the liver appeared enlarged, congested, splenomegaly, congestion in the kidney , hemorrhage in abdominal cavity and Congested gills ,this is in agreement with Umbreit and Tripp.;(1975). In general all the clinical sings and post mortem finding agreed well with Khalil and Abd El-Latif; (2013) for Mugil capito. Also these results are in agreement with Marzouk et al. (2009) for, Sea bass and Sea bream. Prevalence of Vibrio spp. infection: The total number of infected fish was 171 from 311 with 54.98 % prevalence rate. For Sea bass, the total number of infected fish was 72 from 118 with 61 % prevalence rate, For Sea bream, the total number of infected fish was 47 from 97 with 48.45 % prevalence rate, For Mugil capito, the total number of infected fish was 52 from 96 with 54.17 % prevalence rate, these results nearly agreed with El-Gendy (2013) who recorded 44.1% prevalence of isolated microorganism from Seabream and Seabass. In contrast to the current result was slightly lower than 69.9% prevalence of isolated microorganism from Seabream as recorded by Zorrilla et al. (2003); Akayli et al., (2008) and Moustafa et al. (2010). This difference in prevalence might be attributed to different localities and species variation.

For Sea bass, the highest infection rate was recorded during summer season with prevalence rate 100% followed by winter 73%, spring 56% and the lowest was autumn with prevalence rate 36% , For Sea bream, the highest infection rate was recorded during

summer season with prevalence rate 82.61 % followed by winter 58.33 %, spring 42.11 % and the lowest was autumn with prevalence rate 30.23 % , For Mugil capito, the highest infection rate was recorded during summer season with prevalence rate 80 % followed by spring 69.23 %, winter 54.54 % and the lowest was autumn with prevalence rate 40.38 % , the current results were in accordance with that reported by Sabir et al. (2012) who recorded 70. 2% prevalence rate also this result was higher than those reported *by* Hussain (2002); Zorrilla et al. (2003) and El-Adawy (2010); whom mentioned that the total prevalence of Vibrio alginolyticus was 14.61%.

The current results recorded that high prevalence of bacterial infections was correlated with high temperature recorded in summer season and the lowest was recorded in winter season, as reported by Moustafa et al. (2010); Nagib (2011) and Sabir et al. (2012). This can be explained by higher temperatures reduced immune capability and decreased resistance to infection so fish become susceptible to septicemic diseases (Lawson 1995).

The morphological and biochemical properties *of Vibrio alginolyticus and V. parahaemolyticus* from all samples were observed and indicated that the isolated strains are gram-negative, rod-shaped and motile, producing catalase and oxidase and fermentative bacteria that is in agreement with Eleonor et al. (1997); Buller (2004); Liu et al. (2004); Austin (2007)*;* Marudhupandi et al., (2017); Ghenem and Elhadi (2018) and Patel et al .,(2018)

Vibrio parahemolyticus appeared as green colored colonies on TCBS agar due to hemolytic action of the genus while Vibrio alginolyticus appeared as large yellow convex colonies on TCBS agar as described by TWEDT et al.; (1969); Ghenem and Elhadi (2018) and Patel et al .,(2018) .

Antimicrobial (Antibiogram susceptibility testing): The current results of *Vibrio alginolyticus* and *Vibrio parahemolyticus* revealed sensitivity to ciprofloxacin (Cipro5µg), novobiocine (NV30µg). Moreover intermediate sensitivity was found to oxytetracycline (OT 30 µg). While resistance was observed to amoxicillin (Aml 10 µg) and cholistine sulphate (CT 10 µg) as reported by Baumann et al. (1971); Richard and Lhuillier(1977); Ghenem and Elhadi (2018) and Patel et al .,(2018).

The amplified patterns obtained by PCR with tested *V. alginolyticus* strains. All isolates were positively reacted to the collagenase gene primers. Each strain gave almost a common band with the same molecular weight .The three isolates yielded a single band of amplified product specific and clear band of the suspected size (737bp), internal fragment of the collagenase gene primers these results were agreed with the findings of Lajnef et al. (2012), Marhual et al. (2010) and Di Pinto et al. (2005); where the amplified patterns obtained by PCR with tested *V. parahemolyticus* strains. All isolates were positively reacted to the pR72H gene primers. Each strain gave almost a common, specific and clear band of the suspected size (387bp), internal fragment of the pR72H gene primers as agreed with *Lee et al. (1995); Robert-Pillot et al. (2002)*; *Bermúdez-Almada et al.(2014); Li et al. (2016); Chen et al.(2017)* and *Ghenem and Elhadi (2018);* whom demonstrated that amplification of the pR72H fragment, for amplicons of 387 bp, is a powerful tool for reliable identification of *V. parahaemolyticus* which observed in the different strains.

Conclusion: Vibriosis is considered the most important threatening disease problem facing aquaculture specially Seabass, Seabream and Mugil capito.

5. REFERENCES

Abdel-Aziz M., Eissa A., Hanna M. and Abou Okada M., 2013: Identifying some pathogenic Vibrio/Photobacterium species during mass mortalities of cultured Gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax) from some Egyptian coastal provinces. International Journal of Veterinary Science and Medicine, 1(2) 87-95

Abd El-Galil, M.A. and M. H. Mohamed, 2012: First isolation of Vibrio alginolyticus from ornamental Bird Wrasse fish (Gomphosus caeruleus) of the Red Sea in Egypt. Journal of Fisheries and Aquatic Science, 7 (6):461-467.

Abd-Elghany S. M. and Sallam K. I., 2013: Occurrence and molecular identification of Vibrio parahaemolyticus in retail shellfish in Mansoura, Egypt .Food Control, 33(2) 399-405

Adebayo-Tayo, B.C.; I.O. Okonko; M.O. John; N.N. Odu; J.C. Nwanze and M.N. Ezediokpu, 2011: Occurrence of Potentially Pathogenic Vibrio Species in Sea Foods Obtained from Oron Creek. Advances in Biological Res., 5 (6): 356-365.

Akayli, T.; G. Timur; B. Aydemir; A.R. Kiziler; O. Coskun; G. Albayrak and E. Arican, 2008: Characterization of Vibrio alginolyticus Isolates from Diseased Cultured Gilthead Sea Bream, Sparusaurata. The Israeli Journal of Aquaculture – Bamidgeh, 60: 89-94

Anzaldi, L.L., Skaa, E.P. 2010: Overcoming the heme paradox: heme toxicity and tolerance in bacterial pathogens. Infect Immun., 78: 4977–4989.

Arlene J. Chen, Nur A. Hasan, Bradd J. Haley, Elisa Taviani, Mitch Tarnowski, Kathy Brohawn, Crystal N. Johnson, Rita R. Colwelland Anwar Huq (2017). Characterization of Pathogenic Vibrio parahaemolyticus from the Chesapeake Bay, Maryland, doi: 10.3389/fmicb.2017.02460

Austin, B. and D.A. Austin, 1987: Bacterial Fish Pathogen; Disease in farmed and wild fish. John and Sons Chichester

Austin, B. and D.A. Austin, 2007: Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish. Fourth edition ed. Chichester, UK: Praxis Publishing Ltd; p. 594 p. Lee K.K. 1995 Pathogenesis Studies on Vibrio alginolyticusin the Grouper, Epinephelus-Malabaricus, Bloch-EtSchneider. Microbial Pathogenesis, 19: 39-48.

Austin, B. and D.A. Austin, 2012: Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish. Springer Science + Business Media Dordrecht 2012.

Baumann et al., 1971: Vibrio campbellii classification , Published in: Int. J. Syst. Bacteriol. 31::217

Bermúdez-Almada, M.C.; A. Espinosa-Plascencia; M.L. Santiago-Hernández;C.J. Barajas-Borgo and E. Acedo-Félix, 2014. Behavior of Oxytetracycline in Shrimp Cultured Litopenaeus vannamei and sensitivity to three antibiotic of bacteria of Vibrio isolated from the organisms. Biotecnia., 3: 29-37.

Buller, N.B., 2004: Bacteria from fish and other aquatic animals: A practical identification Manual, CABI Publishing, Wallingford, UK. 361

CDC 2016: National Enteric Disease Surveillance: COVIS Annual Summary, 2014. Centers for Disease Control and Prevention, Atlanta, GA, USA.

Cheng, W.; L. Wangb and J. Chen, 2005: Effect of water temperature on the immune response of white shrimp

Litopenaeus vannamei to Vibrio alginolyticus. Aquaculture, 250: 592-601.

Di pino, A.; G. Ciccarese; G. Tantillo; D. Catalano and V.T. Forte, 2005: ACollagenase-Targeted Multiplex PCR Assay for Identification of Vibrioalginolyticus, Vibrio cholerae, and Vibrio parahaemolyticus. Journal of Food Protection, 68(1): 150–153.

Eissa, I. A. M.; H.I. Derwa; M. El-Lamei; A. Desuki; M.S. Zaki and H. ElSheshtawy, 2013: Iron in water and some marine fishes in relation to vibriosis at Lake Temsah. Life Science Journal 10 (3): 2520-2528.

Eleonor, V.A.; L.A. Tendencia and Dureza, 1997: Isolation of Vibrio species from Penaeusmonodon (fabricious) with red disease syndrome.Aquaculture, 154: 107-114.

Elliot E. L., Kaysmer A. C., Tumplin, H. L., 1992: Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnifllus and other Virbio species, in: Bacterial analytical manual, edited by GJ. Jackson, (AOAC International, Arlengton, VA), 111-140.

Ellis A.E. 1999: Immunity to bacteria in fish, 9 (4) 291-308.

El- Adawy, M.M., 2010: Studies on bacterial pathogens affecting Mugil capito fish. Thesis, M. V. Sc. (bacteriology) Fac. Vet. Med., Suez Canal University.

Eleonor, V.A.; L.A. Tendencia and Dureza, 1997: Isolation of Vibrio species from Penaeusmonodon (fabricious) with red disease syndrome. Aquaculture, 154: 107-114.

El-Hady Maha. A., El-Katib Nahla R. and Essam S. Abdel-Aziz 2015: Microbiological studies on Vibrio species isolated from some cultured fishes .Animal Health Research Journal 3 (1):12-19

El-Gendy, M.Y., 2013: Epizootiological and molecular studies on the common septicemic bacterial infections of some salt water fishes. PhD thesis, fish diseases and management, Faculty of Veterinary Medicine, Cairo University, Egypt, 212 pp.

Fryer J. L. , Rohovec J. S., Tebbit, G. L. Mcmichael, J. S., Filcher K. S., 1972: Vaccination for Control of Infectious Diseases in Pacific Salmon, Fish Pathology 10 (2) : 155-164

Hassanein, 2007: Studies on some problems facing shrimp culture in Egypt.PhD Thesis, Fish diseases and management, Faculty of veterinary Medicine, Zagazig University.

Honda, T. and Iida, T. 1993: The pathogenicity of Vibrio parahaemolyticus and the role of the thermostable direct heamolysin and related heamolysins. Rev. Med. Microbiol., 106–113

Hussain, R.A., 2002: Studies on some bacterial infections affecting certain marine fishes in the Arabian Gulf of Kingdom of Saudi Arabia. M.V.Sc. Thesis, Fac. Vet. Animal Res. King Faisal University

Jale, K. and T. Gulsen, 2008: Marine Vibrios associated with diseased sea bass(Dicentrarchus labrax) in Turkey. Journal of fisheries Sciences, 21 (1):66-76.

Khalil, R.H. and H.M. Abd El-Latif, 2013: Effect of Vibrio alginolyticus on Mugilcapito. Journal of the Arabian Aquaculture Society, 8 (1): 193- 204.

Koneman, E. W.; Allen, S. D.; Janda, W. M.; Schreckenberger, P. C. and Winn, C. W. Jr. 1992: Color Atlas and Textbook of diagnostic Microbiology, 4th Edition. JB Lippincott Company, Philadelphia.

Labella, A.; C. Berbel; M. Manchado; D. Castro and J.J. Borrego, 2011:

Photobacterium damselae subsp. damselae an Emerging pathogen affecting New Cultured Marine Fish Species in Southern Spain. Recent Advances in Fish Farms, 9: 135-152.

Lajnef, R.; M. Snoussi; S. Balboa; A. Bastardo; H. Laabidi; A. Chatti; H.Abdennaceur and J. Romalde, 2012. Molecular typing of Vibrio alginolyticus strains isolated from Tunisian marine biotopes by two PCR-based methods (ERIC and REP). African Jour. of Microbio. Research, 6(22): 4647- 4654.

Lawson, T.B. 1995: Fundamentals of Aquaculture Engineering. Chapman and Hall, New York. 355pp.

Lee, C.Y.; S.F. Pan and C.H. Chen, 1995: Sequence of a cloned pR72H fragments and its use for detection of Vibrio parahaemolyticus in shell fish with PCR. Applied and Environmental Microbiology.61: 1311–1317.

Letchumanan V, Yin W-F, Lee L.H, Chan K-G. 2015: Prevalence and antimicrobial susceptibility of Vibrio parahaemo-lyticus isolated from retail shrimps in Malaysia. Frontiers in Microbiology. 6:33. doi:10.3389/fmicb.2015.00033.

Levin M. A. , Wolke R. E., Cabelli V. J., 1972: Vibrio anguillarum as a cause of disease in winter flounder (Pseudopleuronectes americanus), https://doi.org/10.1139/m72-242.

Liu, P .C.; J.Y. Lin; W.H. Chuang, K.K. Lee, 2004: Isolation and characterization of pathogenic Vibrio harveyi (V-carchariae) from the farmed marine cobia fish Rachycentron-canadum L. with gastroenteritis syndrome. World Journal of Microbiology and Biotechnology, 20: 495-499.

Liu P.C., K.K. Lee, 1999. Purification and partial characterization of a toxic serine protease produced by pathogenicVibrio alginolyticus. Microbios. 98 (390): 95-111

Ghenem l. and Elhadi N. 2018: Molecular characterization and antibiotic resistance patterns of Vibrio parahaemolyticus isolated from coastal water in the Eastern Province of Saudi Arabia, J Water Health. 2018 Feb; 16(1):57-69. doi: 10.2166/wh..

Lucky, Z., 1977: Methods for diagnosis of fish diseases. Amerind Publ. Co. PV.Ltd., New York, 1st Ed.

Marhual, N.P.; B.K. Das; M. Sadique; A.K. Swain; B.K. Mishra; N.K. Maitiand A.E. Eknath, 2010. Molecular identification and typing of Vibrio alginolyticus and Vibrio parahaemolyticus strains isolated from black tiger shrimp Penaeus monodon. J. Aquaculture in Trop., 25: 25-33.

Macfadyen, G. et al. 2011: Value-chain analysis of Egyptian aquaculture. Project report. The World Fish Center. Penang, Malaysia. 84pp

Marzouk, M.S.; M.I. Hanna and M.K. Kenawy, 2009: Monitoring the Cause of Mortality in some Marine Fishes in Matrouh Governorate, Egypt during the summer 2008. American- Eurasian J. Agric. and Environ. Sci., 5 (2): 148-158.

Mizuki, H.; S. Whasio; T. Moita; S. Itoi and H. Sugita, 2006: Distribution of fish pathogen Listonella anguillarum in the Japanese flounder hatchery. Aquaculture, 261: 26-32.

Moustafa, M.; A.M. Laila; M.A. Mahmoud; W.S. Soliman and M.Y. El-gendy 2010: Bacterial infections affecting marine fishes in Egypt. J. of American Science, 6 (11): 603-612.

Nagib, T.M., 2011: Polymerase chain reaction for diagnosis of some fish bacterial pathogen. M. V. Sc. Thesis,

bacteriology, Faculty of veterinary Medicine, Zagazig University.

Naka, H.; M. Liu; L.A. Actis , J.H. Crosa, 2013: Plasmid and chromosome encoded siderophore anguibactin systems found in marine Vibrios: biosynthesis, transport and evolution. Biometals., 26 (4): 537-47.

Noga, E.J. 1996: Fish disease Diagnosis and Treatment. Mosby-Yearbook, Inc. St. Louis, MO. 367

Olsson J.C., Joborn A.,Westerdahl A., Blomberg L., Kjelleberg S., Conway P. L. 1998: Survival, persistence and proliferation of Vibrio anguillarum in Juvenile turbot, Scophthalmus maximus (L.), intestine and faeces. Journal of Fish Diseases 21: 1-6

Patel, R.K. C.V. Savalia1, R. Kumar, A.P. Suthar, S.A. Patel,N.G. Patel, I.H. Kalyani and S. Gupta(2018). Determination of Vibrio parahaemolyticus from Retail Shrimp of South Gujarat of Navsari District and their Drug Resistance Pattern, International Journal of Current Microbiology and Applied Sciences, 7(2): 2704-2710

Quinn, P. T.; Markey, B. K.; Carter, M. E.; Donnelly, W. J. and Leonard, F. C. 2002: Veterinary Microbiology and Microbial disease,1st Published Blackwell Science Company, Lowa, State University press.

Reham, A.M. 2009: Some studies on Vibriosis on cultured fish. M.V.Sc. Thesis, Poultry and Fish Diseases, Fac. Vet. Med. Alexandria University

Richard, C., Lhuillier, M. 1977: Vibrio parahaemolyticus et vibrions halophiles: leur importance en pathologie humaine et dans l'environnement, From: Bull. Inst. Pasteur, Paris, p. 345-368, 1977 Translated by the Translation Section Department of the Environment Department of the Environment Fisheries and Marine Service Biological Station St. John's, Nfld. 1978.

Robert-Pillot, A.; A. Guénolé and J.M. Fournier, 2002: Usefulness of R72HPCR assay for differentiation between Vibrio parahaemolyticus and Vibrio alginolyticus species: validation by DNA-DNA hybridization", FEMS Microbiol. Lett., 215: 1-6.

Ruichao Li, Jiachi Chiou, Edward Wai-Chi Chan and Sheng Chen 2016: A Novel PCR-Based Approach for Accurate Identification of Vibrio parahaemo-lyticus, doi: 10.3389/fmicb.2016.00044

Sabir, M.; M.M. Ennaji; B. Bouchrif and N. Cohen, 2012: Characterization of Vibrio alginolyticus Trh Positive from Mediterranean Environment of Tamouda Bay (Morocco). World Environment, 2 (4): 76-80.

Sadek, S. 2013: Aquaculture site selection and carrying capacity estimates for inland and coastal aquaculture in the ArabRepublic of Egypt. In L.G. Ross, T.C. Telfer, L. Falconer, D. Soto& J. Aguilar-Manjarrez, eds. Site selection and carrying capacities for inland and coastal aquaculture, pp. 183–196. FAO/Institute of Aquaculture, University of Stirling, ExpertWorkshop, 6–8 December 2010.Stirling, the United Kingdom of Great Britain and Northern Ireland. FAO Fisheries and Aquaculture Proceedings No. 21. Rome, FAO. 282 pp

Schaperclaus, W.; H. Kulow and K. Schreckenback, 1992: Fish Disease.Vol. 1, 5th Ed., A. A. Balkema / Rotterdam.

Snoussi, M.; H. Hajlaoui; E. Noumi; S. Zanetti and A. Bakhrouf, 2008: Phenotypic molecular characterization of Vibrio alginolyticus strains recovered from juveniles and older Sparus aurata

reared in a Tunisian marine farm. Ann Microbial, 58: 141-146

Tantillo, G.M.; M. Fontanarosa; A. Di Pino and M. Musti, 2004: Updated perspective on emerging Vibrio associated with human infections. Lett. Appl. Microbiol., 39 (2): 117-126.

Twedt, R. M., Peeler, J. T., Spaulding, P.L. 1969: Effective ileal loop dose of Kanagawa-positive V. parahaemo-lyticus. Applied and Environmental Microbiology.

Umbreit TH, Tripp MR. 1975: Characterization of the factors responsible for death of fish infected with Vibrio anguillarum, Can J Microbiol. 21(8):1272-4.

Vecsei, P, Litvek, MK, Noakes, DLG, et al. 2003: A non-invasive technique for determining sex of live North American sturgeons. Env Biol Fish, 68: 333–338

Wedemeyer, Gary,1996: Physiology of Fish in Intensive Culture Systems, ISBN 978-1-4615-6011-1

Yiagnisis, M., F. Athanassopoulou, 2011: Bacteria isolated from diseased wild and farmed marine fish in Greece. Recent advances in fish farms, Dr. FarukAral 1Ed ., 153-168.

Zorrilla, I.; M. Morinigo; D. Castro; M. Balebona and J. Borrego, 2003: Intraspecific characterization of Vibrio alginolyticus isolates recovered from cultured fish in Spain. J. Appl. Microbiol., 95: 1106-1116.