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Detection of Some Virulence Factors of Clinical *V.cholerae* isolates in Najaf /Iraq

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Abstract

Vibrio choleare is widely recognized as an important pathogen associated with diarrheal infections. The current study included the detection of *Vibrio cholerae* which was isolated from the clinical cases of watery diarrhea in Al- Najaf province Teaching Hospital during April 2018 to August 2018. The diagnosis of *Vibrio cholerae* isolates which were diagnosed by (*Culture method, biochemical tests* and Api20E system) , In Culture method was isolated (40) isolate on blood agar and TCBS media and (35) isolates by biochemical tests , While Api20E kit was the important method for diagnosis, which has led to isolate and diagnosis of (30) isolate of *Vibrio cholera*. The results revealed the ability some of *Vibrio cholerae* isolates to produce Some Virulence Factor's such as protease(83%) , lipase(73%) , phospholipase(76%) as well as Haemolysin production(100%).

Keywords: *Vibrio cholerae*, Protease, Lipase , Phospholipase, Hemolysin , Virulence factors

INTRODUCTION :

Vibrio cholera ,the causative agent of cholera is an acute diarrheal infection caused by ingestion of food or water contaminated with the bacterium *Vibrio cholera* that belongs to genus *vibrio*, family *Vibrionaceae* [1]. *Vibrio choleare* have a number of factors which help it to reach and colonize the epithelium of the small intestine and produce a variety of extracellular products that have deleterious effects on euokaryotic cells [2]. Studies of the bacterium have elucidated a number of products important for its virulence, including cholera toxin (CT), whose action is largely responsible for the host secretory response, and toxin-co-regulated pili (TCP), which greatly enhances colonization of the intestinal epithelium [3,4]. A toxin-co-regulated pilus has been shown to be necessary for colonization in both the classical and El Tor biotypes of *V.cholerae* O1[5]. TCP also serves as a receptor for CTXØ phage so it appears that TCP pathogenicity island is the initial genetic element required for the virulence associated genes in *V. Cholerae* [6,7,8]. Besides CT and the pilus, other factors including those which are necessary for survival of the bacteria in vivo, penetration of the mucous layer and adherence to the underlying epithelial cells of the intestine, binding and internalization of CT, evasion of the host defense system, etc., may also contribute to the virulence of this important human pathogen [9]. These factors included other potential toxins, accessory colonization factors, outer membrane proteins, proteases, hemolysins, hemagglutinins (HAs), and in some strains, a capsular polysaccharide, all of which may contribute to survival and multiplication of *V.cholerae* within the host [5,10].

Lipases and lecithinases are also produced by different strains of *V.cholerae* and may be related to the hydrolysis of lipid barrier in intestinal epithelial cells [11,12].While Neuramindase, is secreted to cause an increase in the number of receptors in the gut [13]. *V. cholerae* is a highly motile organism and displays chemotactic behavior during its interaction with the intestinal mucosa [14]. However, the importance of motility in virulence has been subject to somewhat discrepant conclusions [15, 16].. Previous research on the virulence of nonmotile strains led to conflicting results about the importance of motility in virulence[12]. The present study is carried out to achieve the following objectives:

1- Isolation of *V.cholerae* isolates among the clinical cases and identification by API20E system .

2- Detection the virulence factors of *V.cholerae* isolates such as hemolysin, lipase, protease and Phospholipase.

MATERIALS AND METHODS

1- Samples collection: A total of samples collection (150) samples were obtained from the clinical cases of watery diarrhea of patients in Al- Najaf province Teaching Hospital during April 2018 to August 2018 who attended to Bacteriology laboratory in Sciences faculty during this period. They were swabbed onto thiosulfate citrate bile salts sucrose (TCBS) agar and MacConkey agar (MC) the plates were incubated overnight.

2- Identification of *V. cholera* : Morphological colonies characteristics were recorded on microscopic properties by Gram's stain was used to examine the isolated bacteria for studying the microscopic properties such as gram reaction, shape and motility and the media that are used (MacConkey agar, blood agar and TCBS agar) for primary identification of *V.cholerae*. While Biochemical tests used Oxidase test, Catalase test (Hydrogen Peroxide 3%), Simmon's Citrate test, Indole Production test, Motility test were all these tests and urease test result according to [17,18]. Also, API20E system was carried out according to the procedure of (Biomerix company, France).

Detection Virulence Factors of *V. cholera:*

- **Medium of Protease Activity Assay:**

A- Composition:

Solution A: 10 g skim milk was added to 90 ml of distilled water then volume was completed to 100 ml. Gently heated at 50˚C, then autoclaved, Cooled to 50-55˚C.

Solution B: 2 g of agar powder was added to 100 ml of distilled water, mixed thoroughly, then autoclaved, Cooled to 50-55˚C.

B- Preparation:
Aseptically, 100 ml of solution A was mixed with 100 ml of solution B. Poured into sterile Petri dishes, then stored at 4˚C until to use. This media used to detect the ability of the bacteria to produce protease [19].

- **Medium of Phospholipase Activity Assay:**

It has been prepared by dissolving 2.4 g of nutrient agar in 100 ml of distilled water with 1 g of NaCl. After autoclaved and cooled to 50˚C, the addition egg yolk of one egg was done in aseptical condition. Then mixed well and poured into sterile Petri dishes and stored at 4˚C until to use through 24-48 hours [20].

- **Rhan Media of Lipase Activity Assay:**

It has been prepared by dissolving the following materials in 900 ml of distilled water. These materials as following: 5 g of K2HPo4, 5 g of (NH4)2Po4, 1 g of Cacl2. 6H2o, 1g of Mgso4. 7H2o, 0.001 g of Fecl2.6H2o, 0.001 g of NaCl, 20 g of agar powder and 5 ml of olive oil. Then the volume was completed to 1000 ml, final pH was adjusted to 7.2, then autoclaved, cooled and poured in sterile Petri dishes and stored at 4˚C until to use. This media used to detect the ability of the bacteria to produce lipase [21,12].

- **Blood Haemolysis:**

Heamolysin was detection according to[22]. Tested for β-hemolytic activity on base agar (Himedia, India) supplemented with 7% sheep erythrocytes. A Loopful of 18-24 hr cultured growth of TSA was transferred and streaked on blood agar and was incubated for 24 hr at 37 ºC. Zones of Haemolysis around the colonies indicated the ability of these bacteria to haemolyse RBCs [23].

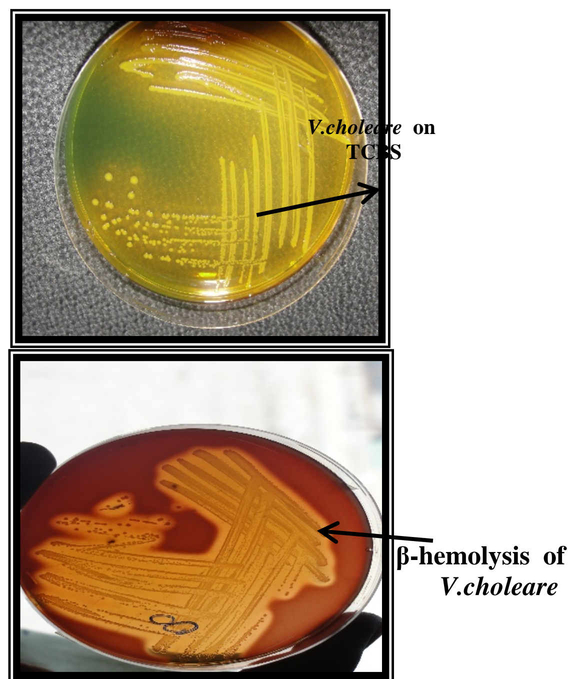
RESULTS AND DISCUSSION

Isolation and Identification

Culture and Biochemical Tests

The isolation and identification of *Vibrio isolates* showed that only 40 isolate were positive based on the morphological characteristics of the colonies on TCBS, MacConkey agar and blood agar media These isolates were smooth yellow, shiny, flat, about 2-3 mm in diameter colonies on TCBS (Fig.1), while they were small and pale colonies on MacConkey's agar when incubated for 24h. While microscopic examination of cultures showed that the bacteria were gram-negative non-spore forming, slightly curved rods arranged as single or double of bacteria and the comma shape or vibriod shape distinguish this bacteria from other gram- negative bacilli. These characteristics were obtained also by previous studies [24,25].

On the other hand, the results of biochemical tests referred to that not all were positive to oxidase and catalase tests. The positive isolates were characterized with the ability to ferment the glucose only on KIA, so the isolates gave alkaline slant with acid bottom without H2S or CO2 production. Also, isolates showed positive results to cholera red, string test and simmon's citrate and negative to urease test(Tab.1). According to these biochemical tests only 33 stool samples showed positive result as *V. cholerae* . This result was predicted by previous studies [8,18,26].



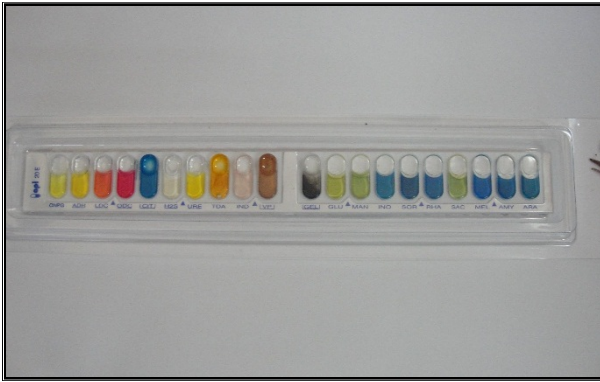
Figure(1): *V. cholerae* isolates colonies on TCBS and blood agar media.

Table (1): Biochemical tests results of *V. cholerae* isolated from stool samples.

Test	Result
Catalase	+
Oxidase	+
String test	+
Methyl red test	+
Urease test	-
Cholera red	+
Citrate test	+
VP	+
Urease	_
String	+
simmone citrate	+
Glucose and lactose Fermentation on KIA	A/K *, No gas/ No H_2S

* A: Acid, K: Alkaline, KIA: Kligler Iron Agar .

In this study API 20E system was used to confirm identification of *V. cholerae* included in this study. The results are shown in (figure 2) That results were in compatible with [27] who observed from 93 collection samples 51 exhibited sucrose-fermenting colonies on TCBS agar and an oxidase-positive reaction. Ninety-six percent of these presumptive isolates revealed excellent identification as *V.cholerae* with the API 20E system and 4% were as a good identification. The remaining 42 strains were not identified. Vieira [28] stated that it was a good evidence to use API 20E system to obtain excellent identification reach up to 99.9% accuracy of the clinical isolates as *V.cholerae* and differentiated from other close bacteria.



Figure(2): API 20E system used in this study.

The isolation and identification of *Vibrio* isolates showed that o gical characte the glucose only on KIA, So the isolates gave alkaline slant with acid bottom without H2S or CO2 production. Also, isolates showed positive results to cholera red, string test .and simmon's citrate and negative to urease test . So, according to these biochemical tests only 35 stool samples showed positive result *as V. cholerae* (Fig.3). This result was predicted by [26, 29,30]. For more assertion the identification of the isolates was done by using API 20E diagnostic kit, the results showed that only 30 of stool samples were positive as *V. cholerae* (Fig.3). This result was predicted by [31,32].

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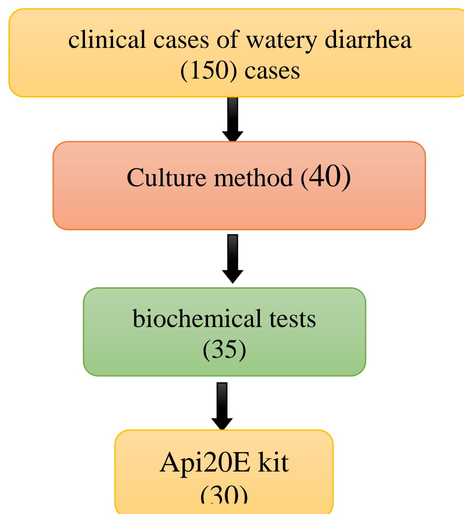

Figure (3): The distribution of *V.cholerae* isolates according to type of diagnosis method.

Detection of Some Virulence Factor's of *V. cholerae*:

- **Protease**

It is necessary to investigate the virulence factors of *V.cholerae* isolates which may a play role in its pathogenicity, so the detection of proteases, lipases and Phospholipase production were done. In this study (30) isolates were examined(Fig.4).. It seemed that (83%) of the clinical isolates of *V.cholerae* produced protease (fig.6). Al-Khafaji, [33] referred to that all isolates in her study were have ability to protease production as well as found (50%) of isolates were protease positive. Proteases produced by *V.cholerae* had a very important role in its pathogenicity, due to the hydrolysis of several physiologically important protein such as mucin, fibronectin, and lactoferrin [34]. It could also proteolytically activate cholera toxin A subunit, El Tor cytolycin and haemolysin [35]. While Abbass, [36] who found that (72.7%) of isolates were protease product and that this enzyme have limited effect on the pathogenesis of this bacteria.

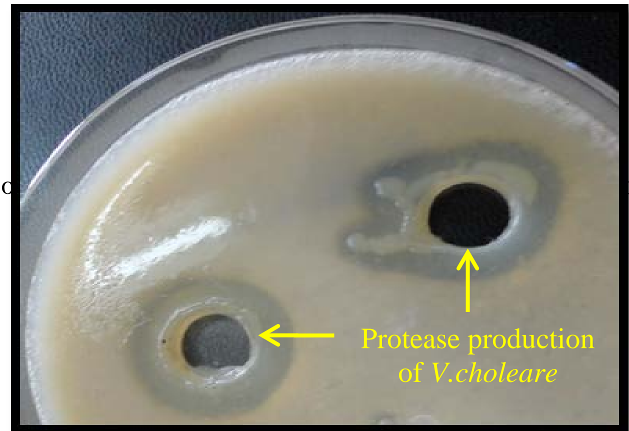

Figure (4): The distribution of Protease enzyme production of *V.choleare*

- **Phospholipase:**

The result showed that the isolates were (76%) produced phospholipases (,fig.4 and fig.6). Japik, [37] referred that (67%) of isolates were have the ability to phospholipase production(Fig.5).. West et al., (2004) found that all isolates were phospholipase positive. Oliver and Kaper, [38]. (2007) mentioned to the role of this enzyme in the cholera disease by the release of Arachidonic acid from the phospholipid found in the cell membranes of the lumen cells, this play an important role in the prostaglandin E2 (PGE2) which is responsible for the increase of liquids secretion from the lumen cells and this lead to watery diarrhea occurs.

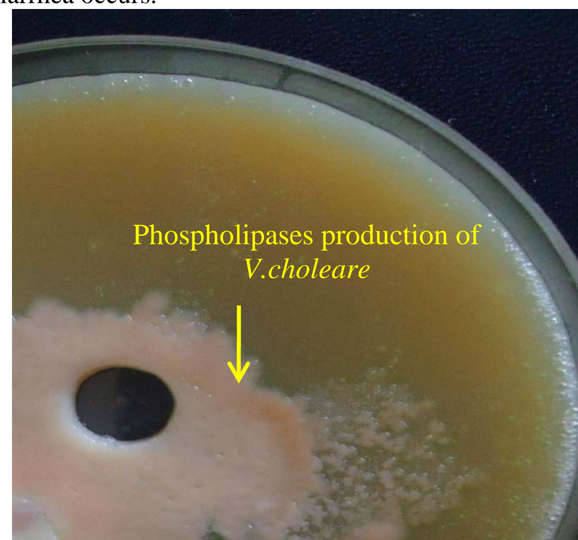

Figure (5): The Phospholipase enzyme production of *V.choleare*

- **Lipase:**

The result showed that (73%) of isolates were have the ability to produce lipase (Fig. 6). Al-Khafaji, [33] referred to that all isolates in her study were have the ability to lipase production. Ingole *et al*.,(1998) found that (65.4%) of isolates were positive to lipase production, also that found (47%) of isolates were lipase positive. Lipases enzymes catalyse the hydrolysis of the ester bonds of triacylglycerols and may have a critical role in *V.cholerae* pathogenicity or nutrition acquisition. The production of excesses amount of lipases allow bacteria to penetrate fatty tissue with the consequent formation of abscesses. Several studies showed that environmental strains of *V.cholerae* may produce a variety of enzymes including proteases, lipases, and haemolysin and other which are necessary for their survival in environment [4, 39].

The production of these enzymes by the isolates may reflect the presence of genetic organization of a discrete genetic element which encodes three genes responsible for the production of proteases, lipases and phospholipase. This organization could be a possible part of pathogenic island, encoding a product capable of damaging host cells and being involved in nutrient acquisition.

- **Haemolysin:**

V.cholerae that investigated through this study was tested for its ability to produce haemolysis. The result of this test showed that this organism able to produce the haemolysin. The appearance of clearly hallo zone around the colony on blood agar medium referred to the ability of this organism to produce the Haemolysin enzyme type beta (β). Microorganisms evolve a number of mechanisms for the acquisition of iron from their environment. One of them is the production of Haemolysin enzyme, which acts to release iron complex [8,40]. The Haemolysin was initially purified by Honda and Finkelstein in 1979 and shown to be cytolytic for the erythrocytes and mammalian cells in culture. The Gene encoding this hemolysin, was hlyA and presented in El Tor, and non-O1 strains of *V.cholerae* [41]. Halpern and Izhaki, [1]. stated that the purified Haemolysin is capable of causing fluid accumulation in ligated rabbit ileal loops .In contrast to the watery fluid produced in response to CT, the accumulated fluid produced in response to Haemolysin was invariably bloody with mucous.

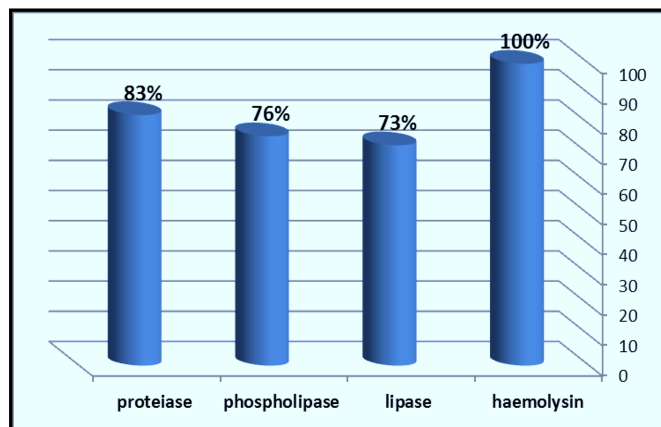

Figure (6): percent of enzyme production of protease, phospholipase and lipase from *V. cholerae* isolates

CONCLUSION:

The following conclusions are extracted from the present study: 1- The frequency of *V. cholerae* from the clinical cases in Najaf (Iraq) and identification by API20E system. 2- Most clinical isolates produced many virulence factors involve heamolysin, lipase, protease, phospholipase**.**

REFERENCES:

[1] - Halpern, M and Izhaki,I.(2017). Fish as Hosts of *Vibrio cholerae*. Frontiers in Microbiology. 8:282.doi: 10.3389/fmicb.2017.00282.

[2]- Matthey, N. and Blokesch, M.(2016). "The DNA-Uptake Process of Naturally Competent Vibrio cholerae". *Trends Microbiol*. 24 (2): 98–110. PMID 26614677. doi:10.1016/j.tim.2015.10.008.

[3]- Herrington, D. A.; Hall, R.H.; Losonsky, G.; Mekalanos, J.J.; Taylor, R.K. and Levine, M.M. (1988). Toxin, toxin-coregulated pili, and the toxR regulon are essential for Vibrio cholerae pathogenesis in humans. J. Exp. Med. 168: 1487–1492.

[4]. Sakib S. N; Reddi.G,a and Almagro-Moreno.S.(2018). Vibrio JB Special Issue. Minireview Environmental role of pathogenic traits in *Vibrio cholerae*. J. Bacteriol. doi:10.1128/JB.00795-17 . 2018 American Society for Microbiology.

[5]. Moore S, Thomson N, Mutreja A, Piarroux R.(2014). Widespread epidemic cholera caused by a restricted subset of *Vibrio cholerae* clones. Clin Microbiol Infect.;20(5):373–9. pmid:24575898.

[6]. Kaufman, M.R.; and Taylor, R.K. (1994). The toxin- coregulated pilus: Biogenesis and function.Chapter 13 In Vibrio cholera and cholera: Molecular to global perspectives. Ed. Wachsmuth, K.; Blake, P.A.; and Olsvik, O.:pp.187-201.

[7]. Siriphap A, Leekitcharoenphon P, Kaas RS, Theethakaew C, Aarestrup FM, Sutheinkul O, et al. (2017) Characterization and Genetic Variation of *Vibrio cholerae*Isolated from Clinical and Environmental Sources in Thailand. PLoS ONE 12(1): e0169324. https://doi.org/10.1371/journal.pone.0169324.

[8]. AL-Fatlawy, H.N.K; Aldahhan,H.A and Alsaadi,A.H.(2017). Phylogenetic of ERIC-DNA Fingerprinting and New Sequencing of Aeromonas Species and V. Cholerae DNA. American Journal of Applied Sciences 2017, 14 (10): 955.964. DOI: 10.3844/ajassp.2017.955.964.

[9]. Kirn TJ, Lafferty MJ, Sandoe CM, Taylor RK.(2000). Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by *Vibrio cholerae*. Mol Microbiol.35(4):896–910. pmid:10692166.

[10]. Al-Fatlawy,H.N.K and AL-Hadrawy,H.A.(2014). Isolation and Characterization of A. hydrophila from the Al-Jadryia River in Baghdad (Iraq). *American Journal of Educational Research, 2014, Vol. 2, No. 8, 658-662*. DOI:10.12691/education-2-8-14.

[11]. Fiore, A.E.; Michalski, J.M.; Russell, R.G.; Sears, CL.; and Kaper, JB. (1997). Cloning, characterization, and chromosomal mapping of aphospholipase (lecithinase) produced by Vibrio cholerae. Infect.Immun. 65 (8): 3112- 3117.

[12]. Pride, A.C; Guan,Z andTrent,M.S.(2014). Characterization of the *Vibrio cholerae* VolA Surface-Exposed Lipoprotein Lysophospholipase. *J. Bacteriol.* 2014, 196(8):1619. DOI: 10.1128/JB.01281-13.

[13]. Perez, M.; Garcia, H.M.; Perez, J.L.; Ceder, B. and Garcia,L. (1997). Obtencion Y purification de neuramindasa de Vibrio cholerae O1. Biotechnologia Aplicada, 14: 256-261. (Abstract).

[14]. WHO, 2016. Microbiological agents in drinking water. Vibrio cholerae. World Health Organization.

[15]. Yancey, R. J.; Willis, D.L. and Berry, L.J. (1979). Flagella-induced immunity against experimental cholera in adult rabbits. Infect. Immun. 25:220–228.

[16]. AL-Fatlawy,H.N.K and Al-Ammar,M.H.(2013). Study of Some Virulence Factors of Aeromonas Hydrophila Isolated from Clinical Samples (Iraq). International Journal of Science and Engineering Investigations, Volume 2, Issue 21, October 2013. Paper ID: 22113-16.

[17]. WHO.(2002). World Health Organization ,Microbiological agents in drinking water. *Vibrio cholerae* (2 nd ed.).

[18]. Jawetz, E., J.I. Melnick and E.A. Adelberg, (2016). Medical Microbiology. 27th Edn., Appleton and Lange U.S.A.

[19]. Benson, H.J. (2002). Microbiological Applications: Laboratory Manual in General Microbiology. (8th ed). Complete version. McGraw-Hill. U.S.A.

[20]. Dogan, B. and Boor, K.J. (2003). Genetic diversity and spoilage potentials among Pseudomonas spp. Isolated from fluid milk products and dairy processing plants. Appl. Environ. Microbiol. 69 (1): 130-138.

[21]. Rodina, A.G. (1972). Laboratory Methods in Aquatic Microbiology. University Perk Press. Battimor Butter Worths, London.

[22]. Peterson, K. M. and Mekalanos, J.J. (1988). Characterization of the Vibrio cholerae ToxR regulon: identification of novel genes involved in intestinal colonization. Infect. Immun. 56:2822–2829.

[23]. Elliot, E. L.; Kaysner, C. A.; Jackson, L. and Tamplin, M. L. (2001). V. cholerae, V. parahemolyticus, V. Valnificus and other Vibrio spp. In: Food and Drug Administration: Bacteriological Analytical Manual, chapter 9, 8th ed. Edited by Merker, R. L., AOAC International, Gaithersburg, MD.

[24]. Cooper, S. (2001) .Helical growth and the curved shape of Vibrio cholerae. FEMS. Microbiol.Lett.198 (2):123-124.

[25]. Carriero, M.M.; Mendes, M.A.A.; Sousa, R.L. and Henrique-Silva,F. (2016). Characterization of a new strain of Aeromonas dhakensis isolated from diseased pacu fish (Piaractus mesopotamicus) in Brazil. J Fish Dis. 2016 Nov;39(11):1285-1295. doi: 10.1111/jfd.12457. Epub 2016 Feb 5.

[26]. Choopun, N.; Louis, V.; Huq, A.; and Colwell, R.R. (2002). Simple Procedure for Rapid Identification of Vibrio cholerae from the Aquatic Environment. Appl. Environ. Microbiol. 68: 995–998

[27]. Baron, S.; Chevalier, S. and Lesne, J. (2007).Vibrio cholerae in the Environment: A Simple Method for Reliable Identification of the Species. J. Health Popul. Nutr. 25(0):312-318.

[28]. Vieira, V.; Teixeira, L. M.; Vicente, A. P. ; Momen,H; and Salles, C.(2001) .Differentiation of Environmental and Clinical Isolates of Vibrio mimicus and Vibrio cholerae by Multilocus Enzyme Electrophoresis . Applied and Environmental Microbiology. Vol. 67: p.2360-2364.

[29]. Al-Hadrawy,H.A.N.(2012). A Comparative study of Bacteriological and Molecular *Vibrio Cholera* Isolated from the Tigris and Euphrates. Ph. D. Thesis, College of the science , University of Kufa. In Arabic .

[30]. Praveen,P.K.; Debnath, C.; Shekhar, S.; Dalai, N and Ganguly, S. (2016). Incidence of Aeromonas spp. infection in fish and chicken meat and its related public health hazards: A review, Veterinary World, 9(1): 6-11.

[31]. Prescott, L.M.; Harley, J.P. and Klein, D.A. (1990). Microbiology. W.M.C.B Brown Publisher, U.S.A.

[32]. Yang Q-H, Zhou C, Lin Q, Lu Z, He L-B,Guo S-L. (2017). Draft genome sequence of *Aeromonas sobria* strain 08005, isolated from sick *Rana catesbeiana*. Genome Announc 5:e01352-16. https://doi.org/10.1128/genomeA.01352-16.

[33]. Al-Khafaji, K. A.A.(2007). Identification of Some Virulence Factors in Toxigenic Clinical and Environmental Isolates of Vibrio cholerae. M. Sc.thesis. Genetic Engineering and Biotechnology. University of Baghdad.

[34]. Finklestein, R. A.; Boesman, M.; Chang, Y.; Hase, C. (1992). Vibrio cholerae Hemagglutinin / protease, colonial variation, virulence, and detachment. J. Infet. Immun. (2): 472 – 488

[35]. Booth, B. A.; Boesman- Finkelestein, M.; and Finkelestein, R. A. (2014). Vibrio cholerae hemagglutinin/ protease nick cholera enterotoxin. Infect. Immun. 45: 558- 560.

[36]. Abbass, N. B.M. (2006). Effectiveness of some physical and chemical factors on the morphological changes of Vibrio cholerae isolated from environment. Ph.D.Thesis, College of Science, University of Al Mustansyria.

[37]. Jabik, N. A. (2000). Study of some Genetic Aspects of Isolated V. cholerae in Babylon. M.Sc.Thesis, College of Science, University of Babylon.

[38]. Oliver, J. D. and Kaper, J. B. (2007) Vibrio Species. In: Food Microbiology: Fundamentals and Frontiers. Edited by Doyl, M. P.; Beuchat, L. R. and Montville, T. J., ASM press, Washington D C., USA. Pp. 228-60 .

[39]. Namdari, H.; Klaips, C.R.; and Hughes, J.L. (2016) .A cytotoxin-producing strain of Vibrio choleraeNon-O1, Non-O139 as a cause of cholera and bacterimia after consumption of raw clams.J. Clin. Microbiol.38 (9):3518-3519.

[40]. Pulungsih, S.P.; Punjabi, N.H.; Rafli, K.; Rifajati, A.; Kumala, S Simanjuntak, C. H.; Yuwono; Lesmana, M. ; Subekti, D. Sutoto; and Fontaine, O .(2006). Standard WHO - ORS versus reduced – osmolarity ORS in the management OF cholera patients. J. Health. Popul .Nutr.; 24:pp 107 - 112.

[41]. Brown, M. H.; and Manning, P. A. (1995). Haemolysin genes of Vibrio cholerae: presence of homologous DNA in non - haemolytic O1 and haemolytic non - O1 strains. FEMS Microbiol. Lett. 30:197–201.