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Effect of some chemical and physical factors on the growth of *Vibrio cholerae* isolated from patients in Najaf Province/Iraq

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ABSTRACT

The present study aimed to isolate of *Vibrio cholerae* which was isolated from the clinical cases of watery diarrhea in Al- Najaf province Teaching Hospital during April 2017 to March 2018 . The study included Two main parts. The first one was the bacterial diagnosis of isolated bacteria based on relied and diagnostic procedures. The second part concerned the study of the ability of *V.cholerae* to resist some chemical and physical factors. This bacteria was suspected for identification by using bacteriological and biochemical tests, after that it was diagnosed using API 20E. The results revealed the ability of this organism to grow in pH ranged from 4 to 10 but could not grow under or above this range. The optimal pH for growth was 8.5. The results exhibited the ability of this organism to grow in different concentrations of sodium chloride, since NaCl was found to stimulate the growth of V.cholerae and optimal concentration was 2.5%, after that the growth became to decline and no growth was found in a concentration of 6%.

KEYWORDS: *Vibrio cholerae*, pH , API 20E, Nacl. Months .

INTRODUCTION :

Vibrio cholerae is a member of the family Vibrionaceae, a Gram negative, facultative anaerobic and non-spore-forming curved rod .The name Vibrio was derived from the Latin name because these curved rods possess a single polar flagellum and appear to vibrate. However, when this organism grown in the laboratory, it frequently reverts to straight rod morphology about 1.4 – 2.6µm long (Atlas, 1997; Thompson and Swings, 2006), capable of respiratory and fermentative metabolism. Bergey's Manual of Systematic Bacteriology (Moore *et al*., 2014) described four genera within Vibrionaceae including Vibrio, Photobacterium, Aeromonas and Plesiomonas. Members of Vibrionaceae use D-glucose as their sole or primary carbon and energy source and most are oxidase positive (Harris et al., 2012). The Cholera disease is caused by infection of the small intestine by Vibrio cholerae O1 and O139 (Rabbani and Greenough, 1999) which is characterized by severe dehydrating diarrheal condition and is one disease in modern times that is epidemic, endemic and pandemic in nature (WHO, 2002a).

Though rare in developing countries, cholera is still an important infection worldwide (Seas et al., 2000). Outbreaks of cholera cause deaths estimated at more than 120,000 annually worldwide and many more cases each year, of which the vast majority occurs in children (WHO, 2005).

In the last decades, attention to cholera epidemiology increased, as cholera epidemics became a worldwide health problem. Detailed investigation of V. cholerae interactions with its host and with other organisms in the environment suggest that cholera dynamics are much more complex than previously times (Codeco, 2001). Cholera is a serious pandemic diarrheal disease and Iraq is at risk of epidemics spreading from neighbouring countries (Al-Abbassi *et al*., 2005) and many cases were recorded in Iraq as early as 1820 during the 3rd pandemic when cholera first spread to Persia and since then cholera continued to appear in an epidemic form (Devault *et al*., 2014).

During the past three years, serious outbreaks have occurred in different parts of Iraq, the country was affected by a large outbreak in 2007 in which (4696) cases with 24 deaths were reported. The outbreak occurred mainly in north of Iraq, which was first detected in Kirkuk spread to Sulaymaniyah, neighboring provinces and Baghdad (WHO, 2008a). Then the

disease reappeared again in 2008 and caused 341 infection cases. The majority of cases were in Babil (58%), followed by Baghdad (18%) and Karbala (9%). Other provinces in which cholera cases have been reported include Anbar, Basra, Diala, Diwanyia, Misan and Najaf (WHO, 2008b).

MATERIALS AND METHODS

Isolation and Identification of *V.cholerae*

Cultural & Microscopic Characteristics

Cultural characteristics: Morphological colonies characteristics were recorded on the media that used (TCBS, MacConkey agar, blood agar) for primary identification of V.cholerae. Also Kligler's Iron Test by A heavy inoculum was streaked over the surface of the slope of kligler iron agar were unformatted as follows (MacFaddin, 2000). Microscopic properties include Gram's stain was used to examine the isolated bacteria for studying the microscopic properties (Collee *et al*.,1996).

Api- 20E technique:

API 20E system consists of twenty micro tubes containing dehydrated substrates. According to manufacturing company (BioMerieux) Microtubes were inoculated with a bacterial suspension equivalent to (MacFarland tube No 0.5). Metabolism produces color changes either spontaneous or by addition of appropriate reagents. Oxidase test was performed separately using oxidase reagents and reactions were read after 24 hrs according to the standard results and the identification was obtained by referring to the analytical profile.

Effect of chemical and physical factors on the growth of *V.cholerae*:

Effect of NaCl:

The effect of NaCl on the growth of *V.cholerae* was tested by the following steps of method according to MacFaddin (2000):

- First step is Peptone water medium was prepared in tubes (10 ml for each tube) and NaCl was added to each tube at various weights to gain different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0) gm/100ml the pH was adjusted to 8.5 by using NaOH (0.1N) then autoclaved.
- First tube was prepared by using same medium free from NaCl.

- The tubes were inoculated with 0.1ml of freshly grown bacterial suspension and incubated for 20 hrs at 37Cº, then serial of tenth fold dilution (10-1, 10-2, 10-3, 10-4, 10-5, and 10-6) were used in sterile distal water for each concentration.
- With the use of spreader 0.1 ml from the last two final dilutions of bacterial suspension were streaked on the supplemented nutrient agar and TCBS then incubated for 24 hr at 37C°.After the period of incubation, the viable count of bacteria was determined by multiply the number of colonies/plate by the dilution factor to obtain the viable count/ml in original suspension.
- Spectrophotometer was also used to determine the optical density for each concentration to confirm the above experiment.

The Effect of pH:

The effect of pH on the growth of V.cholerae was experimentally determined by preparing a series of pH values ranged from 3.5 up to 11.5 by an increment of 0.5 in peptone water medium. All test tubes were autoclaved and inoculated with 0.1 ml of freshly grown bacterial suspension and incubated for 22 hrs at 37°C then serial of tenth fold dilution (10-1, 10-2, 10-3, 10-4, 10-5, and10-6) were used in sterile distal water for each tube. With the use of spreader, 0.1 ml from the each of last two final dilutions of bacterial suspension were streaked on the nutrient agar and TCBS and incubated for 24 hr at 37C°. After the period of incubation the viable count of bacteria was determined by multiply the number of colonies/plate by the dilution factor to obtain viable count cell/ml in original suspension according to MacFaddin (2000):.

RESULTS AND DISCUSSION

Isolation and Identification:

A total of 100 clinical specimens cases of watery diarrhea in Al- Najaf province. The isolation and identification of Vibrio isolates showed that only 85 stool samples were positive based on the morphological characteristics of the colonies on TCBS, MacConkey agar and blood agar media . Microscopic examination showed that *V. cholerae* was gram negative bacillus, coma shaped, non-capsulated, not spore forming with specific darting movement using hanging drop method. More confirmation tests being recommended for identification of *V. cholerae*. The results of these tests were in accordance with standard characters of this

organism in the (Collee *et al*.,1996) . as well as These isolates were smooth yellow, shiny, flat, about 2-3 mm in diameter colonies on TCBS, while they were small and pale colonies on MacConkey's agar when incubated for 24h. Also, some of isolates showed blood hemolysis on blood agar . 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 Dixit *et al*. (2012); Yousif (2016) and Cooper, (2001).

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)

So, according to these biochemical tests only 85 samples showed positive result as V. cholerae Table (1). This result was predicted by Ingole *et al*. (1998); Choopun *et al*. (2002) and Perilla *et al*. (2003).

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

Test	Result
Catalase	+
Oxidase	+
Cholera red	+
String test	+
Urease test	-
Citrate test	+
Methyl red test	+
VP	+
Urease	–
simmone citrate	+
String	+

Test	Result
Glucose and lactose Fermentation on KIA	A/K *, No gas/ No H_2S

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

For more assertion the identification of the isolates was done by using API 20E diagnostic kit, the results showed that only 80 of stool samples were positive as V. cholerae. This result was predicted by Prescott et al.(1990).

According to the reading of biochemical tests, this study showed that from 100 isolate were positive by culture only 85 isolate was positive as Vibrio cholerae and the other 15 isolate were non Vibrio cholerae. This result was different with results of API 20E diagnostic kit which found that only 80 isolates were positive as V. cholerae. The API 20E system is indeed considered an acceptable method for the identification of the more commonly-occurring members of the family Vibrionaceae (Kaper et al., 1995) even if there are very few reports expressly concerned with the ability of commercial systems to identify members of the genus Vibrio (O'Hara et al. 2003).

Table (2):- API 20E system used for identification of *V.cholerae*.

Test	Reactions/Enzymes	Results
ONPG	β-galacosidase Ortho NitroPhenyl - β D-Galactopyranosidase	+
ADH	Arginine DiHydrolase	-
LDC	Lysine DeCarboxylase	+
ODC	Ornithine DeCarboxylase	+
CIT	Citrate utilization	+
H_2S	H_2S production	-
URE	Urease	-
TDA	Tryptophane DeAminase	-
IND	Indole production	+
VP	Acetoin production (VogesProskauer)	+
GEL	Gelatinase	+

Test	Reactions/Enzymes	Results
GLU	Fermentation/oxidation (Glucose)	+
MAN	Fermentation/oxidation(Mannitol)	+
INO	Fermentation/oxidation (Inositol)	-
SOR	Fermentation/oxidation (Sorbitol)	-
RHA	Fermentation/oxidation (Rhamnose)	-
SAC	Fermentation/oxidation (Saccharose)	+
MEL	Fermentation/oxidation (Melibiose)	-
AMY	Fermentation/oxidation (Amygdalin)	+
ARA	Fermentation/oxidation (Arabinose)	-
OX	Cytochrome -Oxidase	+

In this study showed that the distribution of *V.cholerae* according to one year in Najaf province of Iraq as (Fig.2) that showed the optimum growth of V. cholera is in the warm climate (Stavric and Buchanan, 1995 and Elliot et al., 2001). This piece of information supports the results of this study which revealed that August (17) isolates and July (13) isolates were the best months for isolating of stool isolates of V. cholera . This may be due to its suitable warm climate for the pathogen's growth and multiplication. dissimilarly, Kadhim (2009) and Pal *et al*.(2006) found that the highest V. cholera was in the November and October. Emch *et al*.(2008) showed that the most infection occurred in September, November and October. Jabeen *et al*. (2008) noticed that the highest infection was in September and January.

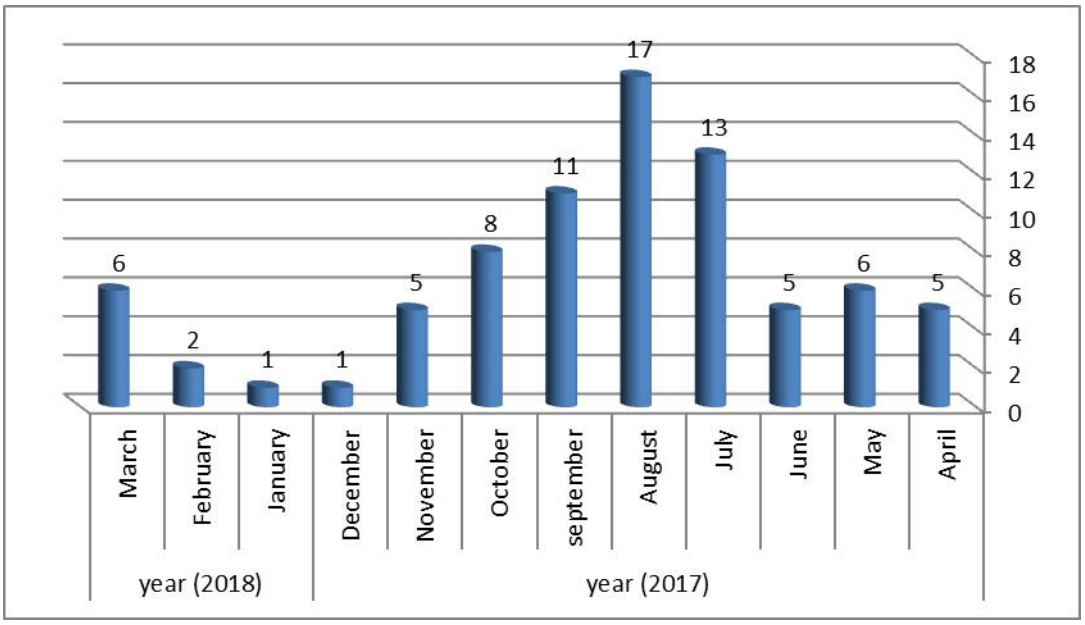

Figure (1): The distribution of V.cholerae according to months of year

Effect of concentration of NaCl on the growth of V.cholerae:

The result showed that the ability of Vibrio cholerae isolates to tolerate different ranges of NaCl. the local isolate *V.cholerae* was able to grow in culture medium without adding NaCl, but the rate of the growth gradually increased to reach the optimum growth at 2.5% NaCl concentration but rate of the growth decreased with an increasing of NaCl concentration to reach the minimum growth at 6% of NaCl concentration. In above At 6.5% NaCl no growth was seen as shown in the figure (3-4). Also the result showed that all isolates failed to grow in the (8%) NaCl concentration .

V. cholerae has been stimulated by addition of 1% sodium chloride. However, an important distinction of *V.cholerae* from other Vibrio spp is the ability of *V. cholerae* to grow in nutrient broth without adding NaCl (Gupta & Chowdhury, 1997), and V. cholerae can grow in a salt range of 0.1–4.0% sodium chloride (ICMSF, 1996).

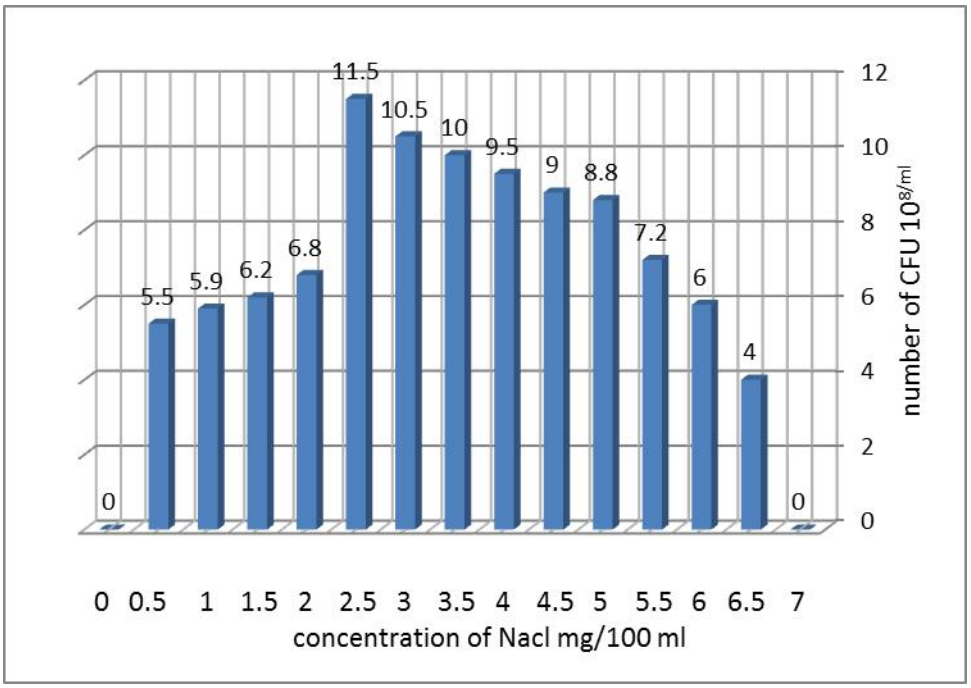

Figure (2):Effect of concentration of NaCl on the growth of V.cholerae

Effect of pH on the growth of V. cholerae:

To study the ability of the isolates to adaptive in this pH value, 0.2ml of the negative tubes was cultured on different media (nutrient agar plates, TCBS and APW). The results of this study revealed that there was a tolerant of this local isolate to some alkaline condition which reachs up to pH 10.0 and the growth began to decline under pH 9.0 and above 8.5, while there was no growth under pH 3 and above pH 11 . These results are agreeable with study by Patel et al., (1995) who found that the survival time of Vibrios in water increased up to 11 days with a highly alkaline pH. As well as Iwanaga et al.,(1986) reported that maximum production of cholera toxin by V.cholerae El Tor strains requires alkaline of medium pH 9.0. Also Al-Karkhy (2005) reported that the best pH for producing protease enzyme was 8.5 at 37° C.

The ability to grow under alkaline conditions is utilized in standard isolation procedures, when samples are pre-enriched in alkaline peptone water (APW) of a pH not less than 8.6 ICMSF (1996).

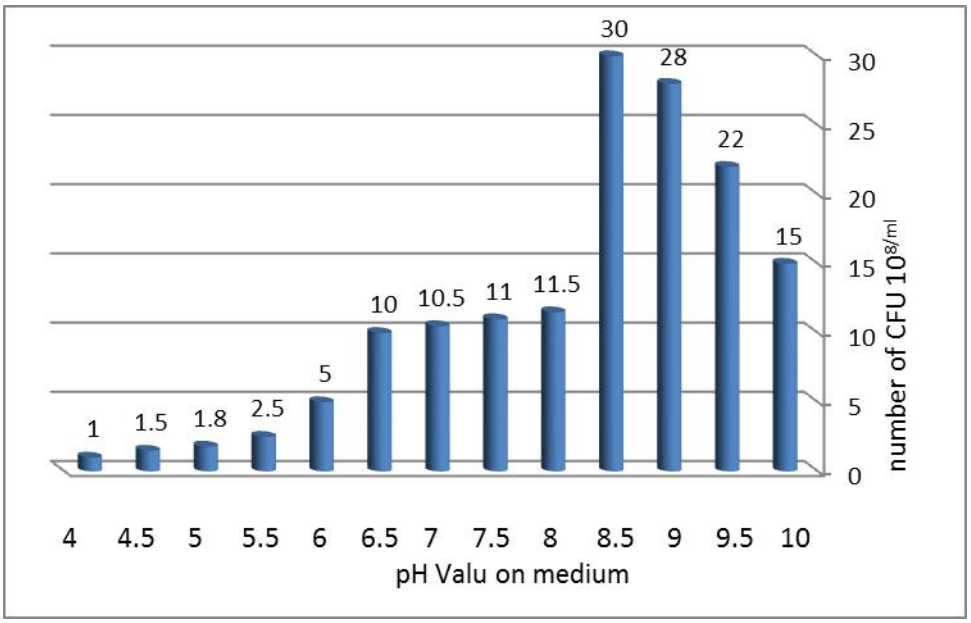

Figure (3):Effect of pH on the growth of V. cholerae V.cholerae

As shown in figure (3-5) *V.cholerae* reached the optimum growth on culture medium when the range of pH between 8.5 and 9.0. In addition, Vibrios are sensitive to low pH and die rapidly in solution below pH 5. however, they are quite tolerant to alkaline conditions. This tolerance was exploited in the choice of media used for the isolation and diagnosis of this organism (Finklestein, 1992).

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