



**University of Babylon
College of Medicine**

**Bacteriological and Serological Study on
Vibrio cholerae – Serovar Inaba in
Babylon Province**

A thesis

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for the Degree of Master in
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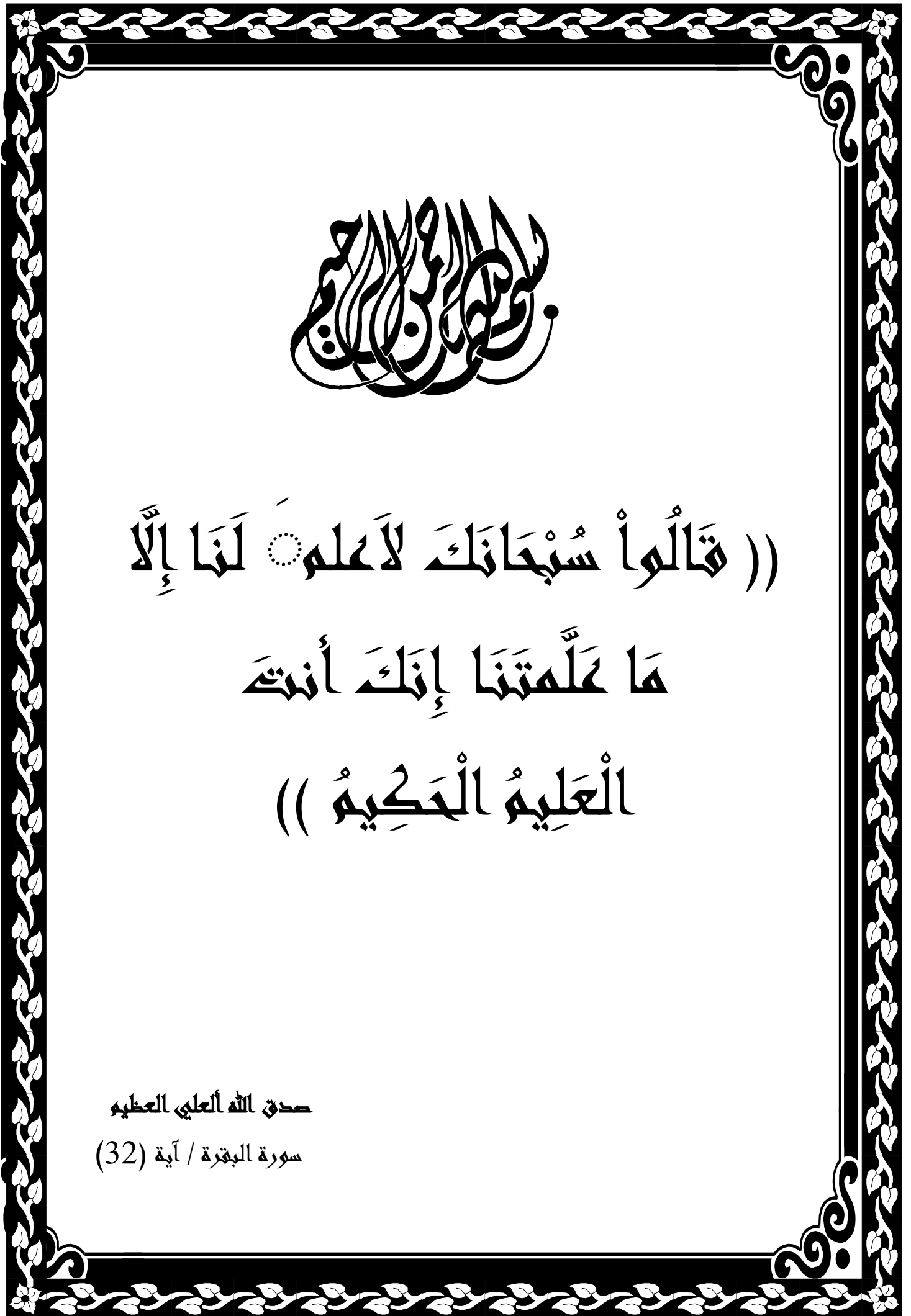
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صدق الله العلي العظيم

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ABSTRACT

The present study executed on one isolate of *Vibrio cholerae* which was isolated from one of the clinical cases of watery diarrhea in Al- Hilla Teaching Hospital during September 2008. The study included three main parts. The first one was the bacterial diagnosis of isolated bacteria based on relied and advanced diagnostic procedures. The second part concerned the study of some virulence factors of *V.cholerae* and the ability to resist some chemical and physical factors. The last part included immunological study including preparation of polyvalent diagnostic antisera and estimation of IgG and IgM level by single radial immunodifution (SRID) test.

Only one bacterium of *V.cholerae* was used in this study. This bacteria was suspected for identification by using bacteriological and biochemical tests, after that it was diagnosed using API 20E, Rapid ID 32E systems and serological tests to detect the serotype of the isolate. From the results of these tests and comparison with specific characteristics, it was found that the isolate of this study was *V.cholerae* Inaba serovare.

The results revealed that this organism was relatively able to grow in a wide range of temperature that ranged from (25 to 45) °C and the optimal temperature was 35 °C but unable to grow above 45 °C. The results revealed the ability of this organism to grow in pH ranged from 5.5 to 10.5 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 3%, after that the growth became to decline and no growth was found in a concentration of 6.5%. Also the bacterium revealed high resistance to garlic oil.

Besides, the results showed that the best concentration of sodium dichloroisocyanurate (NaDCC) commonly used for disinfection of drinking water safety was 5.0 mg/L for 60 min at room temperature with relatively preservative on quality characteristics of drinking water in taste and odor.

The study revealed that *V.cholerae* has some virulence factors represented by producing of Haemolysin type (β), protease enzyme and ability of bacteria to move actively in which enables it to penetrate the mucous layer of intestine in addition to the clinical findings of enterotoxin represented by watery diarrhea.

The results exhibit the resistance of *V.cholerae* to Amoxicillin, Ciprofloxacin and Trimethoprim-sulfamethoxazole but it was sensitive to Tetracycline, Doxycycline, Erythromycin and Chloramphenicol. Tetracycline appeared to be highly active in killing this organism *in vitro*.

Polyvalent diagnostic antiserum has been prepared in two steps and in two different protocols one suggested by this study and the other by Florida State University The somatic antigen was injected in laboratory animals (rabbits). After the fourth injection blood was collected from animal by intracardiac injection then serum has been separated and tested for it's specificity to detect *V.cholerae* using agglutination test. The results were highly positive and specific, since

the specificity of antiserum was determined by slide agglutination test with some types of other bacteria to detect the cross reaction. The results of the two protocols have been compared with commercial foreign antisera and revealed that the protocol suggested by this study was the best in dilution titer which gave 2560 unit/ml and the second protocol gave 1280 unit/ml when compared with commercial foreign antisera which gave 80 unit/ml. Immunological tests showed increase in IgM level in lab animals, rabbit A and rabbit B which gave 3000mg/L and 2400mg/L respectively when compared with control animal (895mg/L). The levels of IgG also increased for lab animals rabbit A and rabbit B which gave 36700mg/L and 37000mg/L respectively when compared with control animal which gave 24500mg/L.

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Fuad

Dedication

*To
The kind hearts*

My father and my mother

My wife

My flowers

Mohammed, Mariam and Amena

To my brothers, my sister with my endless love

Fuad

Supervision Certificate

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List of Abbreviations

Abbreviations	Key
Abs	Antibodies
ACE	Accessory cholera enterotoxin
ACF	Accessory colonization factor
ADP	Adenosine di-phosphate
API	Analytical profile index
APW	Alkaline peptone water
BHIB	Brain heart infusion broth
cAMP	Cyclic adenosine-mono phosphate
CFs	Colonization factors
CIP	Ciproflaxin
CLSI	Clinical laboratory standard institute
DNA	Deoxyribo nucleic acid
GM1	ganglioside receptor
GTP	Guanosine tri phosphate
H ₂ S	Hydrogen sulfide
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KIA	Kligler iron agar

Abbreviations	Complete words of terms
LPS	lipopolysaccharide
LT	Heat-labile enterotoxin
MR	Methyl red
N.A	Nutrient agar
NaCl	Sodium chloride
NAD	Nicotine amide adenine dinucleotide
NaDCC	Sodium dichloroisocyanurate
NAG	Non-agglutinable
NCCLS	National committee for clinical laboratory standards
OMP	Outer membrane protein
PCR	polymerase chain reaction
RBCs	Red blood corpuscles
SIgA	Secretary immunoglobulin A
SPP.	Species
SRBCs	Sheep red blood corpuscles
SRID	Single radial immuno-diffusion
TCBS	Thio sulphate citrate bile salt sucrose
TCP	Toxin co-regulated pilus
WHO	World health organization
ZOT	Zonula occludens toxin

1-1. Introduction:

Cholera, sometimes known as Asiatic or epidemic cholera is an infectious gastroenteritis disease caused by toxigenic *Vibrio cholerae*, which colonizes the small intestine and produces an enterotoxin, cholera toxin. It is responsible for the severe diarrhea (Faruque *et al*, 2006). Since 1817; seven widespread pandemics of cholera have been recorded and still continue to now (Finkelstein, 1996). *Vibrio cholerae* O1 is the etiologic agent of cholera. This serotype is divided into (classical and El Tor) biotype and sub serogroup Inaba, Ogawa and Hikojima.

Non- O1- serovars which belong to different O groups – are called non- agglutinable (NAG) Vibrios (Pourshafie *et al.*, 2002).

A distinctive epidemiological feature of cholera in its appearance in a regular seasonal pattern in areas of endemic infection and in explosive outbreaks often starting in several distinct foci, therefore weather pattern and cholera have been thought to have a link (Colwell, 2004).

United Nation in July - December 2007 reported that a lack of clean drinking water has led to an outbreak of cholera in North of Iraq, in the next year between August and October 2008 another outbreak of cholera emerged in Babylon province which caused mainly by *V. cholerae* O1, Inaba serovars (WHO, 2008).

Infection due to *V. cholerae* begins with the ingestion of contaminated drinking water or food. Pathogens stay in faeces for 7

to 14 days and are shed back into the environment the infected discharge can contaminate the water supply or even the groundwater. However, direct person-to-person spread is rare (Blow *et al.*, 2005).

Cholera begins with sudden onset of acute watery diarrhea that can lead to death by severe dehydration. The patient may lose liter of fluid every hour and associated electrolytes result in cardiac complications and circulatory failure (Sack *et al.*, 2004). Short incubation period - twelve hours to three days enhances the potentially explosive pattern of outbreaks. Cholera is an extremely virulent disease that affects both children and adults, untreated cholera frequently results in high (40-60%) mortality rates (Kaper, 1995). Furthermore *V.cholerae* elaborates several virulence factors that enable it to colonize the gastrointestinal tract and cause the disease. Also, *V.cholerae* produces several enzymes that play important role in the pathogenesis of disease like protease enzyme which has the ability to hydrolyze the mucine and fibronectine also neuraminidase (Janda *et al.*, 1994).

When a cholera case is suspected in an area, a few drops of watery stool or rectal swabs are the most useful specimens which put in a transport medium (Cary-Blair) and sent to the laboratory. The grown organism is transferred to an enrichment medium such as alkaline peptone solution which allows bacteria to grow for 6 to 8 hrs and then cultured on selective medium such as Thio sulphate-citrate- bile salt-sucrose (TCBS) agar. *V. cholerae* could be identified using biochemical reactions (Sack *et al.*, 2004).

To confirm identification *V.cholerae* is subjected to agglutination tests against serogroup-specific antisera. The whole process of *V.cholerae* isolation and identification takes at least 3 days, and by the time the disease may have spread explosively. Therefore, rapid detection of *V.cholerae* is of an important value regarding clinical and financial benefit (Janda *et al.*, 1994).

Rapid management of cholerae cases includes rehydration therapy and replacement of lost electrolytes by an oral rehydration solution or intravenous infusion of fluid in severe cases, in addition to that antibiotic therapy was used in the treatment of cholerae disease (Saha *et al.*, 2006). This management was widely used in Babylon Province through the cholerae outbreak being recorded in summer 2008 particularly in Al-Hashemyia General Hospital, but some real difficulties faced the technicians in all hospitals of the province to diagnose the causes of watery diarrhea rapidly. The main of these difficulties was the storage or absence of the specific diagnostic antisera for *V.cholerae*. The reason that may lead to complicate these difficulties is the import of these antisera from foreign trade.

In an attempt to overcome these difficulties, the objectives of this study were included to prepare the diagnostic antisera in local laboratories as the main object of this study in addition to the following:

- I- Identify of local isolate of *V. cholerae* obtained from stool sample of clinical cases in Al-Hilla Teaching Hospital.

- II- Using API and mini API systems and diagnostic antisera to confirm the identification of above isolate.
- III- Detection of virulence factors (production of haemolysin and protease enzymes) in local isolated *V. cholerae*.
- IV- Investigating the effect of chemical (NaCl, pH, sodium dichloroisocyanurate and garlic oil) and physical factors (temperature) on *V. cholerae* growth.

1-2 Literatures review:

1-2-1 General Description of *Vibrios*:

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 (Holt *et al.*, 1994) 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 (Baumann *et al.*, 1984).

In addition, *Vibrios* are distinguished from enteric bacteria by being oxidase-positive and motile by means of polar flagella in what it is described as darting movement, a diagnostic character for this organism (Brown and Häse, 2001). Most are aquatic microorganisms distributed in fresh water and marine environments (Prescott *et al.*, 1996). Marine *Vibrios* require between 2% and 3% sodium chloride (NaCl) to grow, *V. cholerae* is stimulated by addition of 1% NaCl. However, an important distinction from other *Vibrio* species is the ability of *V. cholerae* to grow in nutrient broth without NaCl (Nair, *et al.*, 1994).

V. cholerae O1, *V. parahaemolyticus* and *V. vulnificus* are the most species that cause disease represented by watery diarrhea also *V. alginolyticus* cause otitis and wound infection. Most strains produce oxidase and catalase enzymes and ferment glucose without producing gas (McLaughlin, 1995). The bacteria are transmitted via contaminated drinking water or foodstuff. It was shown that pathogenic *V. cholerae* can survive refrigeration and freezing in food supplies (Reidl & Klose, 2002).

Moreover, Vibrios survive well in alkaline conditions, but are extremely sensitive to acid and die in solutions with pH less than 6.0. However, the tolerance of *V. cholerae* to alkaline was exploited as a selective factor in the choice of media used for its isolation and diagnosis (Finkelstein, 1996). Additionally more than 200 serogroup of *V. cholerae* species have been identified, but only O1 and O139 contain pathogenic and toxigenic members which cause cholera (Anderson *et al.*, 2004). Most of the strains isolated from cholera patients belong to the O1 serotype. This serotype is divided into three serosubgroups, namely Inaba, Ogawa, and Hijokima. Strains from this serotype were divided into two biotypes, Classical and El Tor. These two biotypes can contain members from all of the serotypes mentioned above (Drasar and Forrest, 1996).

Unlike *V. cholerae* O1 and O139, non - O1/non-O139 typically do not produce cholera toxin, but some strains can cause the disease of watery stool (Ninin *et al.*, 2000).

Farther More, *V. cholerae* can survive environmental fluctuations by its ability to undergo a phase transition, likely in response to

environmental stresses (Colwell and Spira, 1992). There are two morphologically different variants: “Smooth” and “Rugose”. The smooth variant exhibits a smooth and translucent appearance; rugose bacteria on the other hand are wrinkled and opaque (Yildiz and Schoolnik, 1999). Research thus far has shown that the rugose variant shows an increased ability to form biofilms and to deal with environmental stresses than smooth variants. Bacteria in either form can also transit into the other form, with the Rugose to Smooth transition occurring more frequently. Biofilms formation appears to provide some additional protection for bacteria in extreme environmental conditions, and can even make them more resistant to chlorination and antibiotics (Shimada *et al.*, 1994; Faruque *et al.*, 2006).

1-2-2 Epidemiology:

Cholera is a Greek term which means the gutter of the roof as a description of diarrhea of cholerae patients. It is a worldwide disease with an estimated incidence of more than five million cases per year, most of which occur in south East Asia and Africa, with 8% of cases requiring hospitalization. Despite primarily affecting developing countries, cholera remains a serious public health problem for some developed countries (Pazzani *et al.*, 2006). Cholera is a devastating disease, the epidemics of which, until 1992, were caused by *Vibrio cholerae* serotype O1 biotype classical or El Tor. The classical biotype is believed to have caused the first six pandemics, which occurred in the Indian subcontinent and subsequently in other areas of the world between 1817 and 1923.

According to *V. cholerae* O1 biotype El Tor, it was first reported in 1905 at a quarantine camp on the Sinia Peninsula in El-Tor, Egypt by a German physician, E. Gotschlich (Barua & Cvjetanovic, 1972). However, it was not until the early 1960 that *V. cholerae* biotype El Tor displaced the sixth-pandemic *V. cholerae* O1 serotype classical biotype. In 1992 a new serogroup called *V. cholerae* O139 Bengal strain appeared, which was widely spread in Bangladesh, India and its subsequent appearance in Southeast Asia, displacing *V. cholerae* O1 El Tor. It was considered as a significant point in the history of cholera disease. *V. cholerae* O1 El Tor reemerged in 1994 to 1995, but *V. cholerae* O139 continues to coexist with *V. cholerae* O1 as indicated by its temporal quiescence and subsequent reemergence in 1997, 1999, and 2002 (Alam *et al.*, 2006). Epidemics of cholera caused by toxigenic *V. cholerae* O1 and O139 (Bengal strain) represent a major public-health problem in most developing countries (Das and Gupta, 2005). Cholera remains a serious threat in developing countries where sanitation is poor, health care is limited, and drinking water is unsafe (Heidelberg *et al.*, 2000). Hallmarks of the epidemiology of cholera include (i) a high degree of clustering of cases by location and season, (ii) highest rates of infection in children 1 to 5 years of age in areas of endemic infection, (iii) antibiotic resistance patterns that frequently change from year to year, (iv) clonal diversity of epidemic strains, and (v) protection against the disease by improved sanitation and hygiene and preexisting immunity. Cholera has been categorized as one of (Faruque *et al.*, 1998).

1-2-3 Cholerae in Iraq:

In early August 1966 Iraq becomes involved in the seventh cholera pandemic. The disease was transferred from Iran to Iraq (Barua and Burrows, 1974; Kaper *et al.*, 1995).

In 1998 and 1999 explosive out break of cholerae disease occurred and the incidence cases were reached 2376 and 2392 respectively. Most isolates were *V.cholerae* 01, both Inaba and Ogawa serotype in addition to the new serotype (0139) being isolated for the first time in Baghdad and Babylon governorate (Al-Karkhy, 2001; Al-Abbassi *et al.*, 2005).

In addition, the results of study by Al-Karkhy (2003) stated that a total of 100 confirmed cholera cases have been reported in Iraq since high percentage of incidence 48% was occurred in Babylon governorate, 30% in Baghdad governorate and 22% in Basra governorate.

Although, cholera outbreak was detected in Kirkuk, Northern Iraq, on 14 August 2007, it was spread to 9 out of 18 Iraqi provinces. It was estimated that more than 30 000 people have fallen ill with acute watery diarrhoea, among which 3315 were identified as positive for *Vibrio cholerae*, the bacterium causing the disease. A total of 14 people were died from the disease. The case-fatality rate has remained low throughout the outbreak indicating that those who have become sick have been able to access adequate treatment on time (HNSOT, 2008). The disease is continuing to spread across Iraq and dissemination to as yet unaffected areas remains highly possible. Unlike in 2007 the explosive outbreak of cholerae in 2008

was gradually spread. It seems to have started mid August, peaked on September 2008 at 79 cases and then started to fade out during the late of September and October 2008. The majority (53%) of confirmed cholera were reported in Babylon province followed by Baghdad province 17% of cases, Basra 11%, Karbala 8%, Diwanyia 7%, and a total of 4% in Missan, Diala, Najaf and Anbar as shown in the figure (1-1) (WHO, 2008).

In Iraq; the Distribution of confirmed cholera cases was 50% in age between 1-5 years old and 38% in age up to 5 years while only 12% of cases have been reported in other ages as shown in the figure (1-2) (WHO, 2008).

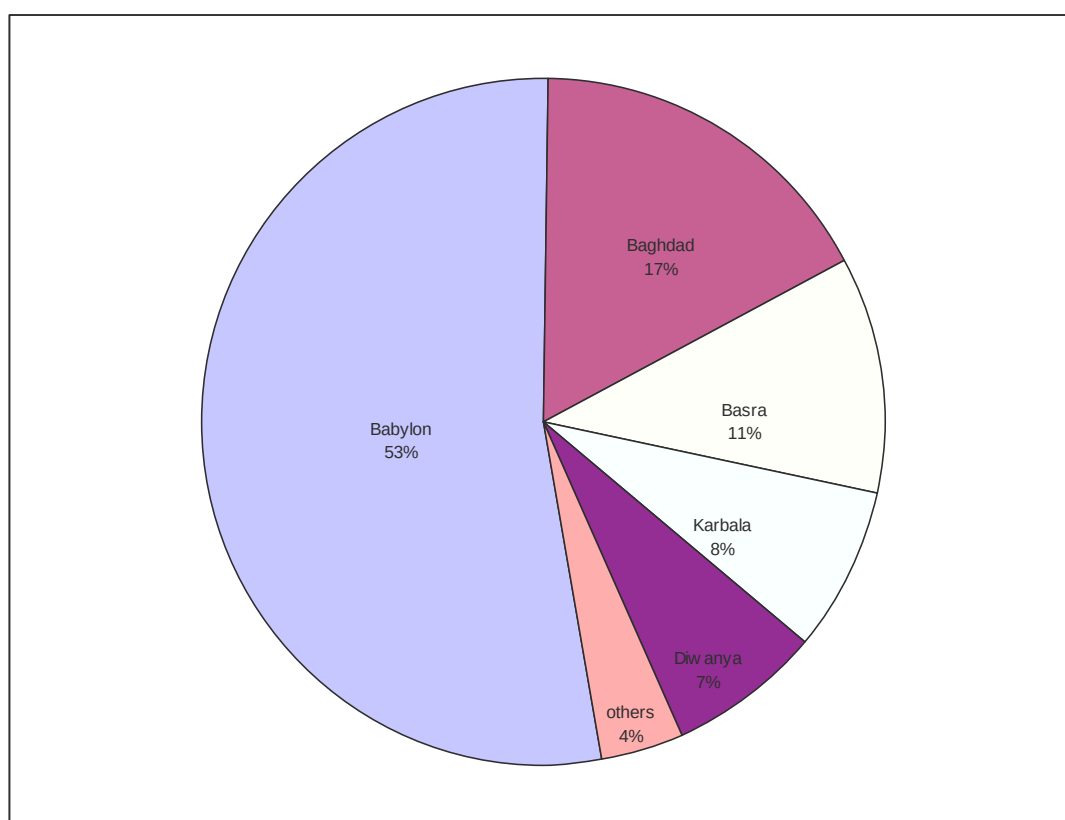


Figure (1-1). Distribution of confirmed cholera by province cases up to 04 October 2008 in Iraq (WHO, 2008).

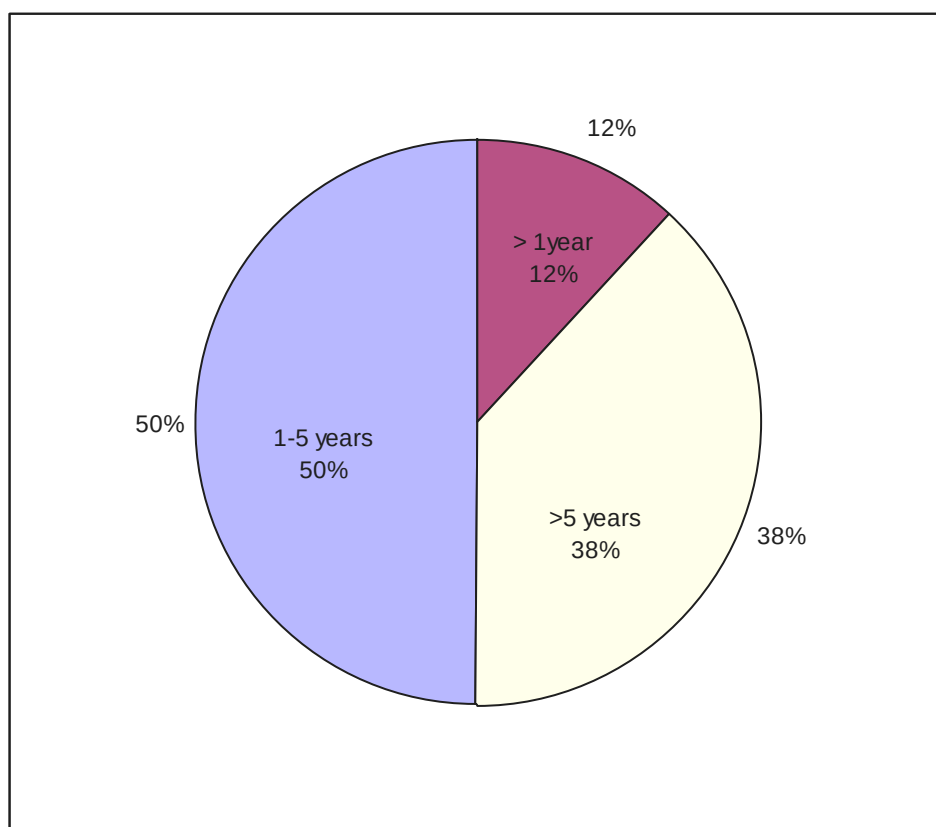


Figure (1-2). Distribution of confirmed cholera cases by age group in Iraq (WHO, 2008)

1-2-4 Antigenic Types & Structures:

Until 1992, the *Vibrios* that caused epidemic cholera were subdivided into two biotypes: classical and El Tor. Classical *V. cholerae* was first isolated by Koch in 1883. Subsequently, in the early 1900s, some *Vibrios* resembling *V.cholerae* were isolated from Mecca-bound pilgrims at the quarantine station at El Tor, in the Sinai, Egypt, that had been established to try to control cholera associated with pilgrimages to Mecca (Alam *et al.*, 2006).

I- Biotyping:

As mentioned above *V. cholerae* O1 strains were divided into two biotypes, classical and El Tor. *V. cholerae* O1 biotype El Tor was first reported in 1905. However, it was not until the early 1960s that *V. cholerae* biotype El Tor displaced the sixth-pandemic *V. cholerae* O1 classical biotype. The vast majority of isolates of the seventh pandemic were of the El Tor biotype (Siddique *et al*, 1991).

The characteristics used to distinguish the classical and El Tor biotypes are given in Table (1-1) (Kaper *et al.*, 1995). El Tor Biotype resemble classical *V. cholerae* in many ways but caused lysis of goat or sheep erythrocytes in a test known as the Greg test. In 1961, cholera caused by El Tor biotype erupted in Hong Kong and spread virtually worldwide (Finklestein *et al.*, 1992).

Table (1-1): Differentiation of classical and El Tor biotypes of *V. cholerae* O1 (Kaper *et al.*, 1995).

Test or Property	Result for biotype	
	Classical	El-Tor
Haemolysin production	(-)	(+)
Agglutination of chicken erythrocytes	(-)	(+)
Inhibition by polymyxin B (50-U disk)	(+)	(-)
Lysis by Classical IV bacteriophage	(+)	(-)

II- Serotyping:

The major surface antigen employed characterization of *V. cholerae* is the O antigen. It is heat stable and is composed of a homopolymer containing the amino acid sugar D-perosamine (4-amino-4, 6-dideoxy-D-mannose) (Manning *et al.*, 1994).

The different O groups are referred to as serogroup or serovars. The rough (R) antigen of *V. cholerae* is identical throughout the species (Shimada and Sakazaki, 1973). The R form is difficult to distinguish from the smooth (S) form by colony morphology alone, but it can be identified by using R antiserum (Sakazaki, 1992).

A flagellar (H) antigen is also present, but the value of this antigen for species identification was limited among all *Vibrio* species (Simonson, and Siebeling, 1988). Unlike other enteric species for which the term serotype usually signifies an assortment of O and H antigens, for *V. cholerae* it usually refers to different antigenic forms of the O1 antigen.

There are multiple classification systems for the O antigen of *V. cholerae*. The most widely used system is the typing scheme of Sakazaki and Shimada, which uses sera against heat-killed organisms. This scheme is made up of 138 different O groups to which the newly described O139 group was recently added (Shimada *et al.*, 1993).

A common feature of all of these systems is the use of the O1 serogroup to signify the serogroup of *V. cholerae* traditionally associated with epidemic cholera. As shown in the figure (1-3) the O1 serogroup was divided into three antigenic forms called Inaba,

Ogawa, and Hikojima. These antigenic forms are referred to as serosubgroups, depending on the reference (Goering *et al.*, 2008).

The O antigen of *V. cholerae* O1 was assumed to consist of three components designated as A, B, and C; the A component may represent the D – perosamine Homopolymer moiety, but the nature of the B and C factors is unknown yet (Stroeher *et al.*, 1992). The differences among these subtypes is largely quantitative; since Ogawa strain significantly contain A and B antigens in addition to a small amount of C. The Inaba strain consist of A and C antigens only (Sakazaki, 1992). The Hikojima contains all three contents. Thereby it crosses reacts with both Inaba and Ogawa antisera. Both Inaba and Ogawa are aggressive pathogens, while the Hikojima subtype is not (Kelly *et al.*, 1991; Sack *et al.*, 2004).

In addition *V. cholerae* O1 strains have been shown to shift between Inaba and Ogawa. This intercom-version is usually irreversible in the laboratory and occurs more frequently in the direction of Ogawa to Inaba (Manning *et al.*, 1994). DNA sequence analysis of gene (*rfb*) encoding the O1 antigen reveals that the sequences for Inaba and Ogawa antigens are nearly identical (Stroeher *et al.*, 1992).

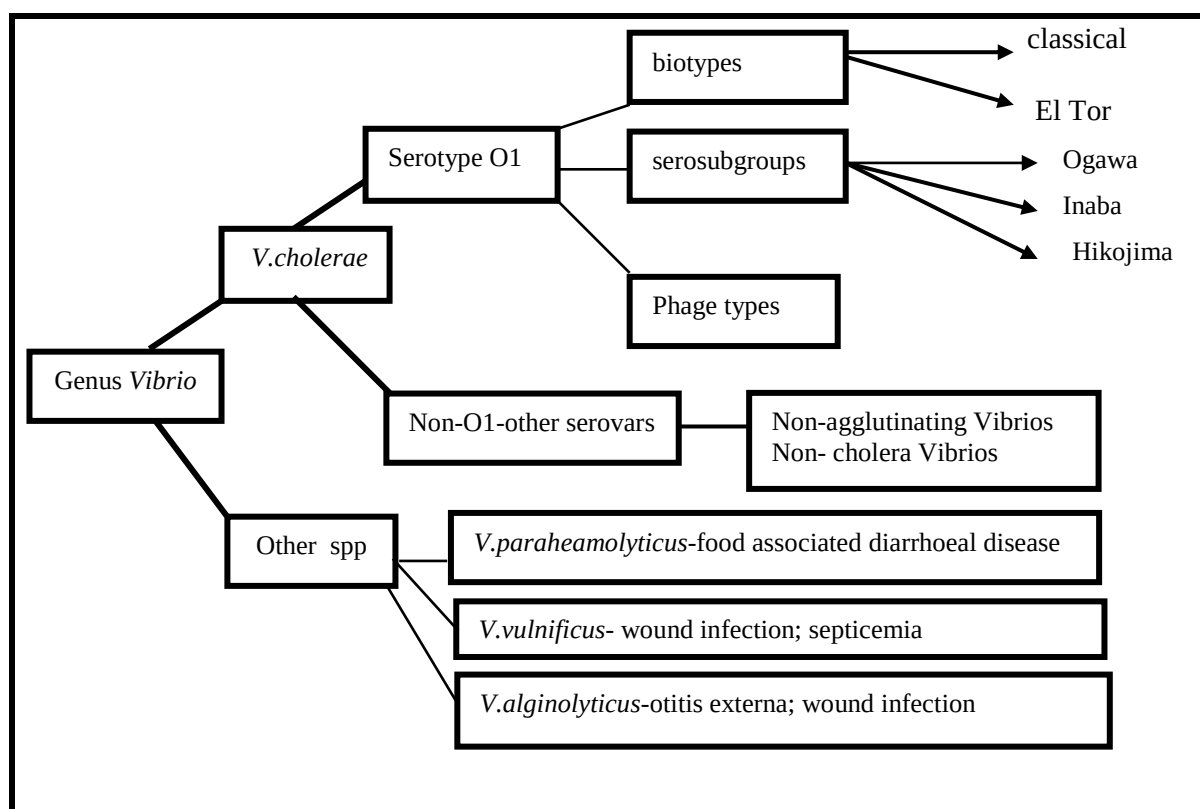


Figure (1-3) *Vibrio cholerae* serotype O1(Goering *et al.*, 2008)

1-2-5 Mode of Transmission of *V.cholerae*:

The general assumption, until quite recently that was cholera spread only by infected people to other susceptible individuals via faecal contamination of water and foodstuff (Guerrant *et al.*, 2000). Recent studies of the aquatic environment, however, have shown that *V. cholerae*, including strains of O1 and O139 are normal inhabitants of surface water, particularly brackish waters, and survive and multiply in association with zooplankton and phytoplankton quite independently of infected human beings (Sack *et al.*, 2004).

The source of the contamination is typically other cholera patients when their untreated diarrhea discharge is allowed to get into

waterways or into groundwater or drinking water supplies. Any infected water and any type foods washed in the water, as well as shellfish living in the affected waterway can cause an infection, while cholera is rarely spread directly from person to person (Griffith *et al.*, 2006).

It has been reported that both toxic and non-toxic strains of *V. cholerae* may exist in water. Non-toxic strains can acquire toxicity through a lysogenic bacteriophage (Jude *et al.*, 2005). Accordingly, *V. cholerae* can be found as free swimming organisms attached to the surfaces provided by plants, filamentous green algae, copepods, crustaceans and insects (Watnick *et al.*, 2001).

Toxigenic strains are mostly isolated from the environment in areas probably contaminated by infected individuals. Environmental isolates from areas that are distant from regions of infection do not generally have the cholera toxin genes (Colwell, 1996).

1-2-6 Pathogenesis and virulence factors:

The major features of the pathogenesis of *V. cholerae* and infection due to this organism begins with the ingestion of contaminated food or water containing the organism (Zhang *et al.*, 1995). After passage through the acid barrier of the stomach, the Vibrios colonize the epithelium of the small intestine. Cholera enterotoxin produced by the adherent Vibrios, disrupts ion transport by intestinal epithelial cells. The subsequent loss of water and electrolytes leads to the severe diarrhea characteristic of cholera (Sack *et al.*, 2004). Colonization aided by way of fimbriae, filamentous protein structures called toxin co-regulated pilus (TCP)

extending from the cell wall, that attach to receptors on the mucosa and bacterium's motility, helps to penetrate the mucus overlying the mucosa (Boyd and Waldor, 2002).

Therefore *V. cholerae* rapidly proliferates- reaching a cell density of $10^7 - 10^8$ cells per g (Sack *et al.*, 2004). Consequently Vibrios closely attached to the mucosa and enterotoxin can be efficiently delivered directly to the mucosal cells. Formerly cholera was thought to cause sloughing of the intestinal mucosa by an inflammatory process. Toxin has the ability to activate adenylate cyclase enzyme in cells of the intestinal mucosa. This activation leads to an increase in cyclic adenosine-mono phosphate (cAMP), followed by an increase in chloride secretion in the crypt cells, and inhibition of neutral sodium chloride absorption in the villus cells, which in turn leads to a massive outpouring of fluid into the small intestine (Todar, 2004) which exceeds the normal absorptive capacity of the bowel and results in watery diarrhea and vomiting. Most of the secretions come from the small intestine, although the toxin inhibits water absorption by the colon (Bashir, 2005).

The diarrhoeal stool contains large amounts of sodium, chloride, bicarbonate, and potassium, but little protein or blood cells (Molla *et al.*, 1981). The loss of electrolyte-rich isotonic fluid leads to blood volume depletion with attendant low blood pressure and shock. Loss of bicarbonate and potassium leads to metabolic acidosis and potassium deficiency (Barua and Burrows, 1974). The stools of cholera patients contain high density of Vibrios (up to 10^8 bacteria per g), therefore they are highly infectious when passed

into the environment they can contaminate water sources and food and may seed an environmental reservoir (Sack *et al.*, 2004).

1-2-6-1 Colonization of the small intestine:

There are several characteristics of pathogenic *V. cholerae* that are important determinants of the colonization process. These include adhesions, neuraminidase, motility, chemotaxis and enzyme production. When the bacteria across and survive the gastric secretions and low pH of the stomach, they are well adapted to survival in the small intestine. *V. cholerae* is resistant to bile salts and can penetrate the mucus layer of the small intestine, possibly aided by secretion of neuraminidase and proteases e.g (mucinases). They withstand propulsive gut motility by their own swimming ability and chemotaxis directed against the gut mucosa (Todar, 2008).

In addition, a specific adherence of *V. cholerae* to the intestinal mucosa is probably mediated by long filamentous fimbriae that form bundles at the poles of the cells. These fimbriae have been termed Tcp (toxin co-regulated pili), because expression of these pili genes is co-regulated with expression of the cholera toxin genes (kirm *et at.*, 2000).

Two other possible adhesins in *V. cholerae* are a surface protein that agglutinates red blood cells (hemagglutinin) and a group of outer membrane proteins which are the products of the acf (Accessory colonization factor) genes (Chiavelli *et al.*, 2001), acf mutants have been shown to have reduced ability to colonize the intestinal tract. It has been suggested that *V. cholerae* might use

these non fimbrial adhesions to mediate a tighter binding to host cells than is attainable with fimbriae alone.

Furthermore *V. cholerae* produces a protease originally called mucinase that degrades different types of protein including fibronectin, lactoferrin and cholera toxin itself. Its role in virulence is not known since mutations in the mucinase gene (designated hap for hemagglutinin protease) do not exhibit reduced virulence. It has been suggested that the mucinase might contribute to detachment rather than attachment. Possibly the Vibrios would need to detach from cells that are being sloughed off of the mucosa in order to reattach to newly formed mucosal cells(Todar, 2004).

1-2-6-2 Cholerae toxin:

In 1959 De and Dutta were the first to demonstrate this toxin by use of culture filtrates in rabbits, now called cholera toxin or choleragen and purification of the toxin 10 years later by Finkelstein and LoSpalluto allowed numerous investigators to discover fundamental properties of the toxin such as structure, receptor binding, and mode of action (Finklestein, 1992).

Cholera toxin and the closely related heat-labile enterotoxin of *Escherichia coli* (LT) are responsible for numerous cases of diarrhea worldwide, leading to considerable morbidity and mortality (Fan *et al.*, 2001). It has a molecular mass of 84 000 Da and consists of five binding (B) subunits and one active (A1) subunit and a bridging piece (A2) that links A1 to the 5 - B subunits. B subunits are physiologically inactive, but bind the

holotoxin to the GM1 ganglioside receptors present on the surface of the small-intestinal mucosal cells (Sack *et al.*, 2004). After cholera toxin binds to intact cells, there is a lag phase of 15 to 60 min before adenylate cyclase is activated. The lag time is necessary to allow the A1 peptide to translocations through the membrane and to come into contact with the G proteins (Lencer *et al.*, 1992; Nambiar *et al.*, 1993).

Once the cholera toxin has bound to the GM1 receptor on host cell, the A1 subunit is released from the toxin by reduction of the disulfide bond that links it to A2, and enters the cell (O`Neal *et al.*, 2005). One hypothesis is that the 5 B subunits form a pore in the host cell membrane through which the A1 unit passes. The bacterium produces an invasion neuraminidase during the colonization stage which has the interesting property of degrading ganglioside to the monosialosyl form, which is the specific receptor for the toxin. After the A subunit is transported into the cell where it is enzymatically transfers ADP ribose from NAD to a protein (called Gs), that regulates the adenylate cyclase system which is located on the inside of the plasma membrane of mammalian cells (Galen *et al.*, 1992).

Enzymatically, fragment A1 catalyzes the transfer of the ADP-ribosyl moiety of NAD to a component of the adenylate cyclase system. The process is complex. Adenylate cyclase is activated normally by a regulatory protein (GS) and GTP; however activation is normally brief because another regulatory protein (Gi) hydrolyzes GTP. The A1 fragment catalyzes the attachment of

ADP-Ribose (ADPR) to the regulatory protein forming Gs-ADPR from which GTP cannot be hydrolyzed as illustrated in (Fig 1-5) (Todar, 2008). Since GTP hydrolysis is the event that inactivates the adenylate cyclase, the enzyme remains continually activated, the net effect of the toxin is to cause cAMP to be produced at an abnormally high rate which stimulates mucosal cells to pump large amounts of Cl^- into the intestinal contents (Gaash, 2005).

Furthermore, H_2O , Na^+ and other electrolytes follow due to the osmotic and electrical gradients caused by the loss of Cl^- . The lost H_2O and electrolytes in mucosal cells are replaced from the blood. Thus, the toxin-damaged cells become pumps for water and electrolytes causing the diarrhea, loss of electrolytes, and dehydration which are the characteristic of cholera (De Haan and Hirst, 2004).

1-2-7 Organization and Regulation of Virulence Factors in *V.cholerae*:

The pathogenesis and production of virulence factors in *V.cholerae* is a multifactorial process and involves several genes encoding cholera toxin (ctxAB), zonula occludens toxin (zot), accessory cholera enterotoxin (ace), hemolysin (hlyA), toxin-coregulated pilus (tcpA), and outer membrane protein (ompU) (Sing *et al.*, 2001). These genes Regulated and aid the pathogen in its colonization, coordinated expression of virulence factors, and toxin action (Vidal *et al.*, 2009). In *V.cholerae*, the major virulence genes

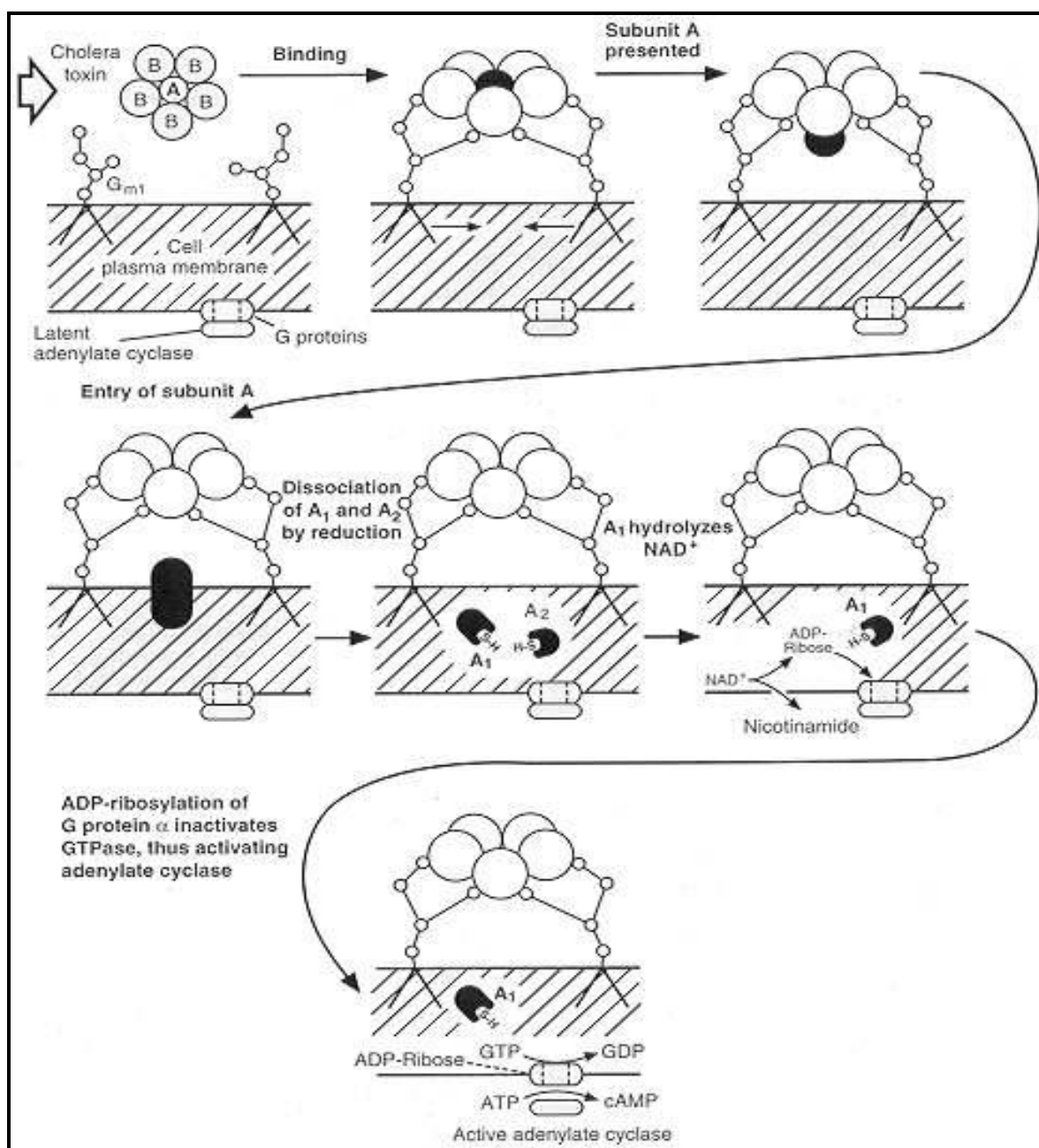


Figure (1-4):- Mechanism of action of cholerae enterotoxin (Todar, 2008).

required for pathogenesis are in clusters and can apparently propagate laterally and disperse among different strains (Waldor and Mekalanos, 1996).

V. cholerae enterotoxin is a product of *ctx* genes. *ctxA* encodes the A subunit of the toxin, and *ctxB* encodes the B subunit. The genes

are part of the same operon. The transcript (mRNA) of the *ctx* operon has two ribosome binding sites (rbs), one upstream of the A coding region and another upstream of the B coding region. The rbs upstream of the B coding region is at least seven-times stronger than the rbs of the A coding region. In this way the organism is able to translate more B proteins than A proteins, which is required to assemble the toxin in the appropriate 1A: 5B proportion (Todar, 2008).

Also, the components are assembled in the periplasm after translation. Any extra B subunits can be excreted by the cell, but A must be attached to 5 B in order to exit the cell. Intact A subunit is not enzymatically active, but must be nicked to produce fragments A1 and A2 which are linked by a disulfide bond.

Transcription of the *ctxAB* operon is regulated by a number of environmental signals, including temperature, pH, osmolarity, and certain amino acids. Several others *V. cholerae* genes are co-regulated in the same manner including the *tcp* operon, which is concerned with fimbrial synthesis and assembly. Thus the *ctx* operon and the *tcp* operon are part of a regulon, the expression of which is controlled by the same environmental signals.

The proteins involved in control of this regulon expression have been identified as ToxR, ToxS and ToxT. ToxR is a transmembranous protein with about two-thirds of its amino terminal part exposed to the cytoplasm. ToxR dimers, but not ToxR monomers, will bind to the operator region of *ctxAB* operon and activate its transcription (Hughes *et al.*, 1994).

ToxS is a periplasmic protein. It is thought that ToxS can respond to environmental signals, change conformation, and somehow influence dimerization of ToxR which activates transcription of the operon. ToxR and ToxS appear to form a standard two-component regulatory system with ToxS functioning as a sensor protein that phosphorylates and thus converts ToxR to its active DNA binding form. Expression of ToxT is activated by ToxR, while ToxT, in turn, activates transcription of *tcp* genes for synthesis of *tcp* pili (Skorupski and Taylor, 1997). Thus, the ToxR protein is a regulatory protein which functions as an inducer in a system of positive control. Tox R is thought to interact with ToxS in order to sense some change in the environment and transmit a molecular signal to the chromosome which induces the transcription of genes for attachment (pili formation) and toxin production (Sack *et al.*, 2004).

1-2-8 Clinical features:

After an incubation period about 12 to 72 hrs, symptoms are generally appearing including watery diarrhea and painless vomiting. The most distinctive feature of cholera, that the stool resembling rice-water with fishy odor, mucus in the stool imparts the characteristic rice water appearance (Finkelstein, 1996). The vomitus is generally a clear, watery and alkaline fluid. In adults with severe cholera, the rate of diarrhea may quickly reach 500–1000 ml/h, leading to severe dehydration (Sack *et al.*, 2004).

In addition, signs of severe dehydration include absent or low-volume peripheral pulse, undetectable blood pressure, poor skin, sunken eyes, and wrinkled hands and feet. At first, patients are restless and extremely thirsty, but as shock progresses, they become apathetic and may lose consciousness (Morris and Jr, 1994). Many patients show respiratory signs of metabolic acidosis with gasping breathing.

Furthermore, stool bicarbonate losses and lactic acidosis associated with dehydration can result in severe acidosis manifested by depression of blood pH and plasma bicarbonate (Kaper *et al.*, 1995). The isotonic dehydration results in serum sodium and chloride concentrations which are usually within the normal range (Wang *et al.*, 1986). Despite profound potassium loss, uncorrected acidosis may result in a normal or high serum potassium level. However, loss of cellular potassium can lead to hypokalemic nephropathy and focal myocardial necrosis. Ischemic renal tubular necrosis due to prolonged circulatory collapse may be seen in patients in whom treatment is long delayed or inadequate (WHO, 1993). Hypoglycemia with coma and convulsions may occur in children (Butler *et al.*, 1999). Most patients have no urine output until the dehydration is corrected. The fluid loss may be so rapid that the patient is at risk of death within a few hours of onset, and most deaths occur during the first day of infection. However, if rehydration fluids are provided in sufficient quantities, the patient may survive safely (Sack *et al.*, 2004).

1-2-9 Diagnosis of *V. cholerae*:

Faeces and rectal swab are the most preferred specimens for isolation of *V.cholerae* during the occurrence of cholera. Whenever possible, stool or rectal swab specimens should be inoculated on isolation plates with minimal delay. Viability of *V.cholerae* is well maintained in "rice-water stool" but is unpredictable in formed stools. Direct demonstration of *V.cholerae* is not recommended for general purposes. However, darting motility using hanging drop method and its inhibition by specific antisera, especially after brief inoculation in broth, has proven valuable in experienced hands for presumptive diagnosis (Chin, 2000). *V. cholerae* is capable of growing on routine laboratory media since the salt content of these media is sufficient to support the growth of these organisms (Forbes *et al.*, 2007). There are several biochemical tests for identification of Vibrios. Moreover, in recent years rapid identical techniques for identification of *V.cholerae* was developed by using Api 20 E and Rapid ID 32 E systems which were developed and produced by Biomerix Company. Serological test one of the most important identification tests for *V.cholerae* by using slid agglutination test (Baron *et al.*, 2007).

1-2-10 Polymerase chain reaction (PCR) for detection of *V.cholerae*:

The polymerase chain reaction method is a molecular technique which provides a highly specific, sensitive, and rapid detection method to facilitate the detection of toxigenic and non

toxigenic strains of *V. cholerae* and therefore play a key role in the cholera surveillance program (Tracz *et al.*., 2007). Attempts to identify *V. cholerae* strains were directed toward the identification of toxigenic strains of *V. cholerae* through the use of cholera toxin gene (*ctxAB*) probes or appropriate primers for the amplification of toxin genes by PCR assay (Nandi *et al.*, 2000).

1-2-11 Treatment:

It has been agreed that the most successful management of cholera is of two parallel ways of therapy; rehydration and antimicrobial therapy and these therapy are described shortly below:

1-2-11-1 Rehydration therapy: This includes rehydration therapy and replacement of lost electrolytes, which contain important ions, such as sodium and potassium, used in biochemical processes to keep the body alive. Rehydration can be accomplished by intravenous infusion of fluid in severe cases or by oral rehydration solution (Swerdlow and Ries, 1992). The hypoglycemia that is sometimes seen in pediatric patients should be treated with 25 or 50% glucagon given intravenously. For adults, the intravenous replacement solution should be infused as rapidly as possible so that about 2 liters is given in the first 30 min. If at this point the patient's clinical condition improves, the infusion can be slowed to deliver approximately 100 ml/kg of body weight within the first 4 hrs of therapy (Kaper *et al.*, 1995).

Also, Children in shock should receive 30 ml of intravenous fluid per kg of body weight in the first hour and an additional 40 ml/kg in the next 2 hrs. The intravenous fluid chosen for rehydration should be adequate to replace the isotonic fluid and electrolyte losses of cholera (Sack *et al.*, 2004).

Patients with mild or moderate dehydration can receive initial fluid replacement to repair water and electrolyte deficits exclusively by the oral route. For mild dehydration, the WHO recommends oral rehydration solution is given in a volume of 50 ml/kg within the first 4 hrs. For moderate dehydration, twice this volume 100 ml/kg should be given in the same time period. Vomiting rarely prevents successful use of oral rehydration solution and is not a contraindication to its use (Pulungsih *et al.*, 2006).

There is much interest in the use of complex carbohydrates such as those found in rice gruel (Zaman *et al.*, 2001). Oral rehydration solution that uses rice rather than glucose is even better. Patients can be fed as soon as they are able to take food. There is no need to restrict food or fluids, and babies can continue to breastfeed (Sack *et al.*, 2004).

1-2-11-2 Antimicrobial therapy:

Antimicrobial agents play a secondary but valuable role in therapy by decreasing the severity of illness and the duration of excretion of the organism (Bennish, 1994).

Tetracycline and Doxycycline are the drug of choice for adults, the dosage of tetracycline 500 mg four times per day for 3 days while the dosage of Doxycycline is 300mg one single dose per day

(Swerdlow and Ries, 1992). Use of tetracycline in children, 8 years of age remains somewhat controversial, because of the possibility of staining of permanent teeth (Alam *et al.*, 2006). The new quinolones (norfloxacin and ciprofloxacin) have excellent in vitro activity against *V. cholerae* and may prove to be useful therapeutic agents (Saha *et al.*, 2005). However, in vitro susceptibility of *V. cholerae* to antimicrobial agents does not always correlate with in vivo activity. Erythromycin for 3 days was recommended for children in a dose of 10 mg/kg twice daily while 250 mg four times per day was recommended for pregnant women (Gilbert *et al.*, 1999). As compared with erythromycin, azithromycin a macrolide antibiotic derived from erythromycin, has better in vitro activity against *V. cholerae* has a longer half-life, and has less gastrointestinal toxicity (HopKins, 1991), also it has shown to be effective when used in a single dose for the treatment of cholera in children (Khan *et al.*, 2002).

1-2-12 Immunity to Cholera:

After natural infection by *V. cholerae*, circulating antibodies can be detected against several cholera antigens including the toxin, somatic (O) antigens, and flagellar (H) antigens (Todar, 2008). These antibodies are also raised by parenteral injection of antigens as vaccine components. Antibodies directed against O antigen are considered "Vibriocidal" antibodies because they will lyse *V. cholerae* cells in the presence of complement and serum components. Vibriocidal antibodies reach a peak within 8-10 days after the onset of clinical illness, and then decrease, returning to the

baseline 2 - 7 months later (Kaper *et al.*, 1995). Although, recurrent infections of cholera are in fact, rare, and this is probably due to local immune defense mediated by antibodies secreted onto the surfaces of the intestinal mucosa, patients who recovered from cholera are solidly immune for at least 3 years (Finkelstein, 1996). Moreover, in children who are nursing cholera is less likely to occur, presumably due to protection afforded by secretory antibody in mother's milk. Secretory IgA, as well as IgG and IgM in serum, can be detected in the intestinal mucosa of immune individuals (Chowdhury *et al.*, 2008). Antibodies against flagella or somatic O antigens could cause clumping and arrested motion of cells. Antitoxic antibodies could react with toxin at the epithelial cell surface and block binding or activity of the toxin. Since the process by which the Vibrios attach to the intestinal epithelium is highly specific, antibodies against vibrio fimbriae or other surface components lipopolysaccharide (LPS) could block attachment of Vibrios to the intestinal epithelium (Apter *et al.*, 1993).

New oral cholera vaccines promise substantial protection without side-effects and highly immunogenic (Anh *et al.*, 2007). A killed whole-cell oral vaccine (Dukoral) consists of killed *V. cholerae* organisms along with the cholera B subunit, and the vaccine therefore stimulates both antibacterial and antitoxic immunity. Two doses are given 1– 6 weeks apart (Legros *et al.*, 1999). The other vaccine (Orochol) is an attenuated virulent mutant of *V. cholerae*, given as a single-dose (Tacket *et al.*, 1999).

2-1 Materials:-**2-1-1 Bacterial isolate:-**

The isolate included in this study was obtained from suspected cholera case referred to the laboratory of Al-Hilla Teaching Hospital and confirmed as *V.cholerae*- serotype O1-serosubgroup Inaba by this study.

2-1-2 Equipments and apparatuses:-

The devices indicted in the list below were used for general purposes throughout the study.

Equipment	Source
Autoclave	HiraYama, Japan
Automatic micropipette	Slamed, Germany
Centrifuge	Hettich, Germany
Electric balance	A&D company, Japan
Eppendrof Centrifuge	Hettich, Germany
Hot air oven	Memmert, Germany
Incubator	Memmert
Inoculating loop & needle	Japan
Light microscope	Olympus, Japan
pH- meter (Multiline)	Weilheim, Germany
Spectrophotometer	Optima, Japan
Water bath	Memmert

2-1-3 Biological and chemical materials in the list below were used throughout the study.

i- Culture Media:

Culture media	Source
Agar-agar	Himedia, India
Api- 20 E	Biomerieux ,France
Blood agar base	Oxoid , England
Brain-Heart infusion agar & broth	Difco , USA
Cary & Blair transport medium	Defco, USA
Gelatin medium	Difco, USA
Kligler iron agar	Oxoid , England
MacConkey agar	Oxoid, England
MR-VP medium	Oxoid , England
Muller- Hinton agar	Oxoid , England
Nutrient agar & broth	Difco , USA
Peptone water medium	Mast , England
Rapid ID 32E	Biomerieux ,France
Simmons citrate medium	Mast, England
Skim milk medium	Difco , USA
Starch agar	Difco, USA
TCBS agar	Biolife, Italy
Urea agar base	Biolife, Italy

ii– Chemical materials:

Chemicals & biological materials	Source
Ethanol (99%) , HCl (37%) , HgCl ₂ NaOH, NaCl, BaCl ₂ , H ₂ SO ₄ (98%), Glycerol, Diethyl ether (98%), Calcium chloride, Glucose, Arabinose, Mannitol, Sucrose	BDH, England
H ₂ O ₂	Fluka, Switzerland
Mineral oil	Biomerieux, France
Garlic oil	Ranbaxy, India
Sodium dichloroisocyanurate (NaDCC)	Medentech, Ireland

2-1-4 Reagents and stains:

Reagents and stains	Source
Gram's stain set	Crescent, Saudi
Oxidase reagent (N, N, N, N, tetra methyl-P-phenylene diamine dihydro chloride)	Fluka, Germany
Kovac's reagent	Himedia, India
(VP) Potassium hydroxide & α naphthol + ethanol	Biomerieux, France
Methyl red	BDH, England
IgM and IgG kits	Binding site, England

2-1-5 Diagnostic antisera:

Diagnostic antiserum	Source
Polyvalent (O1)	Mast, England
Monovalent -Inaba	Mast, England
Monovalent - Ogawa	Mast , England

2-1-6 Antibiotic disks used throughout the study:

Type	Abbreviation	potency	Source
Vibriostatic disc O/129 2,4-diamino-6,7-diiso propyl pteridine phosphate)	DD15	150µg	Oxoid, England
Tetracycline	(TE)	30 µg	Bioanalyse, Turkey
Amoxicillin	(Ax)	25 µg	Bioanalyse, Turkey
Erythromycin	(E)	15 µg	Bioanalyse, Turkey
Polymixin-B	(PB)	100U & 50 U	Bioanalyse, Turkey
Chloramphenicol	(C)	30 µg	Bioanalyse, Turkey
Ciproflaxin	(CIP)	5 µg	Bioanalyse, Turkey
Doxycycline	(Do)	30 µg	Bioanalyse, Turkey
Trimethoprim- Sulphamethazole	(SXT)	1.25+23.75	Bioanalyse, Turkey

2-1-7 Laboratory animals:

Three New Zealand albino (white) females' rabbits were obtained from animal house of Al-Kufa medical collage and used in this study under the same conditions (weight 2.3-2.5 kg and 16 weeks of age). The serum of the three animals were checked by agglutination with *V.cholerae* serotype O1-Inaba lived antigen,

after that two of them were injected intravenously (using the marginal vein) with heat-killed *V.cholerae* serotype O1-Inaba, but in different protocol as in the table (2-1). The third animal was used as control and not injected with *V.cholerae* Inaba antigen.

Table (2-1):- Protocol of injection for the laboratory animals with somatic antigen of *V.cholerae* serotype O1-Inaba (Florida State University, 2007).

Antigen injection	Rabbit (A)		Rabbit (B)	
	Date	Dose	Date	Dose
Initial antigen injection	Day 0	0.5ml	Day 0	0.5ml
First antigen booster	Day 5	1.0ml	Day 7	0.5ml
Second antigen booster	Day 10	1.5ml	Day 21	1.0ml
Third antigen booster	Day 15	2.0ml	Day 35	1.0ml
Blood collection	Day 23		Day 44	

2-2 Preparation of Culture media:

The general culture media described below were prepared by the routine methods and used in appropriate experiments. The pH at all culture media was adjusted to 9.0 except when it is necessary for some media.

2-2-1 Alkaline peptone water (APW):

This medium was prepared by dissolving 10 grams of peptone and 5 grams of (NaCl) in one liter of distilled water and then sterilized by autoclave. This medium was used to enhance the growth of *V.cholerae* at 6-8 hours before inoculated into another media (McLaughlin, 1995).

2-2-2 Blood agar medium (B.A):

This medium was prepared by suspending 40 grams of blood agar base in one liter distilled water. The suspension was heated to dissolve the medium and sterilized by autoclave at 121°C, 15 pound/inch² for 15 minutes. The medium then was supplemented with 7-10% of fresh human blood after cooling to 45°C. This medium was used to enhance the growth of organism and to determine the ability of this organism to produce the Haemolysin enzyme as recommended by MacFaddin (2000).

2-2-3 Cary & Blair transport medium:

This medium was prepared according to manufacturing company by suspend 12.6 grams of powder in 991 ml of distilled water. The suspension was heated with frequently agitation until completely dissolved. The medium was cooled to 50°C and 9 ml of 1% aqueous calcium chloride was added. Then pH was adjusted to 8.4 ±0.2 and dispensed in screw capped test tubes and sterilized by autoclave. This medium was used as transport and preservation media for *V.cholerae* as recommended by (Baron & Finegold, 1990).

2-2-4 Kligler iron agar:

Kligler iron agar was prepared as recommended by manufacturing company and used for determining glucose and lactose fermentation with or without gas production and for detection test of hydrogen sulfide (H₂S) and gas production as a first step in the identification of Gram- negative bacilli as being staked by MacFaddin (2000).

2-2-5 MacConkey agar medium:

MacConkey agar medium was prepared according to the method recommended by the manufacturing company and it was used for the primary isolation of Gram-negative bacteria and differentiation of lactose fermentative from the non lactose fermentative bacteria (Collee *et al*, 1996).

2-2-6 Maintenance medium:

This medium included Brain heart infusion broth (BHIB) supplemented with 25% glycerol. The medium was autoclaved and used for preservation and maintenance of bacteria for relative long time as recommended by Fetham and coworker (1978).

2-2-7 MR-VP medium:

MR-VP medium was prepared according to manufacturing company and used to detect the complete and partial hydrolysis of glucose according to MacFaddin (2000).

2-2-8 Muller- Hinton agar:

Muller-Hinton agar was prepared according to the method recommended by Cruikshank and colleagues (1975). This medium was used in anti-microbial susceptibility testing and Vibriostatic disk (O/129) and for sensitivity of *V.cholerae* to polymyxin-B that was used to differentiate between Classical biotype and EL Tore biotype of *V.cholerae*.

2-2-9 Nutrient agar (N.A):

This medium was prepared according to the manufacturing company. It was used for general experiments.

This medium was used to reactivation *V.cholerae* and prepares it for biochemical and serological test (MacFaddin, 2000).

2-2-10 Semi- solid nutrient medium:

This medium was prepared by adding 0.5% agar agar to nutrient broth. This medium was used for detecting the motility of bacteria regarding to the method recommended by Collee and colleagues (1996). Motile bacteria typically give diffuse hazy growth that spread throughout the stab-line (Murray *et al.*, 2003).

2-2-11 Simmons' citrate medium:

This Medium was used for determining the ability of bacteria to utilize citrate as the sole carbon source (MacFaddin, 2000).

2-2-12 Skim milk agar:

This medium was prepared according to manufacturing company by dissolving 5.1 grams from medium in 100 ml of cold distilled water and was boiled to dissolve the medium completely, then autoclaved. This medium was used to detect the ability of bacteria to hydrolyze the protein.

2-2-13 Starch agar:

This medium was prepared by dissolving 2.5 grams of starch in 100 ml of basal medium (Nutrient agar). The mixture was heated to dissolve the medium completely. The pH was adjusted at 7.2 and then sterilized by autoclave. This medium was used to detect the ability of bacteria to liquefaction the starch by amylase enzyme (MacFaddin, 2000).

2-2-14 Sugar fermentation media:

This medium is composed of two main constituents as follows:

A. Basal medium:

It was prepared by dissolving 10 grams of peptone, 1gram meet extract, 5grams (NaCl) and 0.08 gram phenol red in one liter distal water. The pH was adjusted to 7.0. This medium was equally distributed in test tubes and Durham tube was placed (up side down) in each test tube then autoclaved.

B. Sugar solutions:

These solutions were prepared by dissolving 1 gm of sugar (Arabinose, Manitol, Sucrose, Glucose) in 100 ml distilled water and sterilized by filtration, and then 0.1 ml of each solution was added to each of the above test tube. This medium was used for diagnosis pathogenic bacteria that have the ability to ferment sugar (MacFaddin, 2000).

2-2-15 Thio sulphate citrate bile salt sucrose agar (TCBS):

TCBS agar medium was prepared according to the method recommended by the manufacturing company, that 90 grams of medium were suspended in one liter of distilled water and heated with agitation until boiling and not autoclaved. The medium was cooled in water bath to 50-55°C and then poured in sterilized Petri dish. This medium was used as differential and selective media for *V.cholerae* (Henry, 1996).

2-2-16 Urea agar medium:

This medium was prepared according to manufacturing company, by suspended 2.4 grams urea agar base in 95 ml distil

water and the medium was boiled to dissolve the medium completely. The pH was adjusted at 6.8 then this medium was autoclaved and cooled to 50°C then 5ml of 20% filtration sterilized urea solution was added. The medium was distributed into sterile tube and allowed to set in a slope position. This medium was used for detecting bacterial ability to produce urease enzyme (MacFaddin, 2000).

2-3 Biochemical reagents & chemical solution:

2-3-1 Oxidase test reagent:

This reagent was prepared by dissolving 0.1 gram of tetra methyl-P-phenylene diamine dihydrochloride in 10 ml of distilled water. It was used immediately after preparation (Collee *et al.*, 1996; Brooks *et al.*, 2007).

2-3-2 Catalase test reagent:

This reagent was prepared in 3% concentration of H₂O₂ and it was used to identify bacterial ability to produce catalase enzyme (MacFaddin, 2000).

2-3-3 Kovac's reagent:

This reagent was prepared by dissolving 0.5 gram of P-dimethylamine benzylaldehyde in 7.5ml of amyle-alchole and 2.5 ml of concentrate HCl was added. It was used to detect the indol production (Baron, *et al.*, 1994; MacFaddin, 2000).

2-3-4 Methyl red reagent:

Methyl Red Reagent was prepared by dissolving 0.1gram of methyl red in 300 ml of 95% ethanol and then the volume was

completed to 500 ml by distilled water. This reagent was used to detect the complete glucose hydrolysis (MacFaddin, 2000).

2-3-5 Vogus-Proskaur reagent (VP):

This reagent was prepared according to Collee *et al* (1996). It consisted of two solutions:

- a- α -Naphthol- reagent 5%: It was prepared by dissolving 5gm of α -Naphthol in 100 ml of 99% ethanol.
- b- Potassium hydroxide KOH 40% solution: 40 grams of KOH was dissolved in 100 ml of distilled water. This reagent was used to detect the partial glucose hydrolysis.

2-3-6 McFarland nephelometer standards:

The solution of tube No 0.5 was prepared by mixing 0.05 ml of 1.175% aqueous solution of barium chloride with 9.95 ml of 1% sulfuric acid H_2SO_4 . The mixture resulted in turbidity approximately equal to bacterial cell density of 1.5×10^8 cell/ml (Baron *et al.*, 1990). Correct density of the turbidity standard was verified by reading the absorbance at 625 nm. The absorbance should be 0.08 to 0.10 for the McFarland standard. Tube number 1.0 was prepared by mixing 0.1 ml of 1.175% aqueous solution of barium chloride with 9.9 ml of 1% sulfuric acid H_2SO_4 as recommended by NCCLS (2003).

2-3-7 Aqueous solution of sodium deoxycholate (0.5%):

This solution was prepared by dissolving 0.5 gram of sodium deoxycholate in 100 ml distil water. This solution was used in string test (Cherly *et al.*, 1999).

2-3-8 Frazee's reagent:

The reagent was prepared by dissolving 15grams HgCl_2 in 20ml concentrated HCL, and then 100 ml of distilled water were gradually added. This reagent was used to detect the bacteria ability to hydrolyze gelatin (Collee *et.al.*, 1996).

2-3-9 Normal saline solution:

It was used for suspending bacterial cells and general experiments and it was prepared by dissolving 8.5 gram NaCl in 1000ml of distilled water and autoclaved as recommended by (Cruickshank *et al.*, 1975).

2-4 Laboratory Diagnosis:

According to the diagnostic procedures recommended by (Holt *et al.*, 1994; Baron *et al.*, 1994; Collee *et al.*, 1996; MacFaddin, 2000; Frobes *et al.*, 2007) the isolation and identification of *V.cholerae* were performed as follows:-

2-4-1 Physiological and Biochemical Tests:**2-4-1-1 Oxidase Test:**

This test was performed by transferring a part of bacterial colony by sterilized woody stick on filter paper saturated with recently prepared oxidase reagent. The appearance of dark purple color within 10 seconds indicated for positive result (MacFaddin, 2000).

2-4-1-2 Catalase Test:

Catalase production was detected by emulsifying pure bacterial colonies of 24 hours incubation in 3% H_2O_2 on the

surface of a clean slide. The appearance of gas bubbles was indicated for a positive result (Baron *et al.*, 1994).

2-4-1-3 Indol Test:

A test tube containing a peptone water medium was inoculated with pure colony of the tested bacteria and incubated at 37 C° for 18 hrs then several drops of Kovac's reagent were added to the medium with gentle shaking. The appearance of the red ring on the surface of broth culture was regarded as a positive result (MacFaddin, 2000).

2-4-1-4 Cholera red test:

This test included addition of drops of concentrated H₂SO₄ 98% to overnight growth in alkaline peptone water medium. The appearance of red color was recorded as a positive test (Cruickshank *et al.*, 1975).

2-4-1-5 Methyl Red Test:

The tubes of the (MR-VP broth) were inoculated with the selected bacterial colonies and incubated at 37°C for 24 hrs, then 5 drops of methyl red reagent were added to it. The appearance of red color means a positive result and a complete analysis of glucose (MacFaddin, 2000).

2-4-1-6 Vogus Proskaur Test:

The tubes of (MR-VP broth) were seeded with bacterial culture and incubated at 37°C for 48 hours then 0.6 ml of α-naphthol reagent and 0.2 ml of 40% KOH solution were added. The appearance of red color after 15 min indicates for a positive

result due to partial analysis of glucose, which produces acetone or Acetyl methyl-carbinol (MacFaddin, 2000).

2-4-1-7 Citrate utilization test:

The tube contained slant of Simmon Citrate agar was inoculated with *V.cholerae* by stabbing in the bottom and streaking on the slant and incubated at 37C° for 24 - 72 hours. Changing the color of medium from green to blue indicates for a positive result (Collee *et al.*, 1996).

2-4-1-8 H₂S Production:

Only the colonies growing on TCBS & MacConkey agar were touched by a straight wire and inoculated on Kligler's Iron Agar by stabbing the bottom of the tube and streaking the slant. Fermentation was detected by changing the indicator phenol red to yellow. The result of this test was recorded after 18 - 24 hours of incubation. Gas formation is usually visualized as bubbles in the medium while H₂S production appears as black precipitate in the bottom of the medium (Baron, *et al.*, 1994).

2-4-1-9 Urease test:

The tube that contained a slant of (urea agar) was seeded with tested bacteria and incubated at 37C° for 24 hours. Changing the color of medium to deep pink indicates for positive result (Collee *et al.*, 1996).

2-4-1-10 Motility test:

A test tube contained semisolid media was inoculated with tested bacteria by singly stabbing down the center of the tube to

about half the depth of the medium and incubated at 37C° for 24 - 48 hrs. The presence and disseminating of hazy growth outer the stabbing line indication of motile bacteria (MacFaddin, 2000).

2-4-1-11 Haemolysin production:

The ability of *V.cholerae* to produce Haemolysin enzyme was carried out by streaking the blood agar medium with bacterial isolate. The appearance of clear zone around the colonies indicates for complete haemolysis (β -haemolysis) or greenish zone around the colonies indicates for partial haemolysis (α -haemolysis), while no change in the medium indicates for no-haemolysis (γ - haemolysis) (Fropes *et al.*,2007).

2-4-1-12 Protease production:

This test was carried out by inoculating wells previously were made in skim milk agar with 0.1 ml of bacterial suspension and incubated for 24 hrs. Presence of clear zone around the wells indicates for positive result (Collee *et al.*, 1996).

2-4-1-13 Gelatin test:

The gelatin media plate was seeded by tested organism and incubated under appropriate conditions for (2-3) days and then treated with Frazier's reagent. The positive test resulted in pale zones a round the gelatin liquefying colonies (MacFaddin, 2000).

2-4-1-14 Carbohydrate fermentation test:

Carbohydrate fermentation medium was inoculated with bacterial suspension and incubated at 37C for 24 hrs. Changing the color medium from red to yellow indicates for the ability of bacteria to ferment the sugar under test (MacFaddin, 2000).

2-4-1-15 Starch hydrolysis test:

Detection of amylase production was carried out by inoculating of starch agar medium with bacterial growth and incubated at 37°C for 24hrs. Lugol's iodine was added to this medium. The appearance of clear zone around the growth refers to complete hydrolyzes of starch, while the reagent gives purple-blue color with out hydrolyzed starch (MacFaddin, 2000).

2-4-1-16 String test:

Fresh growth of *V.cholerae* was used for this test from Kligler's Iron Agar. String test was performed on a clean glass slide by suspending over night growth of Kligler's Iron Agar in a drop of 0.5% aqueous solution of sodium deoxycholate. A mucoid “string” was formed when an inoculating loop was drawn slowly away from the suspension which indicates for a positive result (WHO, 1987).

2-4-1-17 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 as shown in table (Appendix -2) and the identification was obtained by referring to the analytical profile.

2-4-1-18 Rapid ID 32E technique:

Rapid ID 32E is a standardized system for the identification of *Enterobacteriaceae* and certain Gram-negative rods which are oxidase positive (*Aeromonas*, *Plesiomonas*, *Acinetobacter*, *Pseudomonas* and *Vibrio*). This system consists of 32 test cupules which contain dehydrated test substrates. According to manufactured company (BioMerieux) microtubes were inoculated with 55µl of bacterial suspension with a turbidity equivalent to (McFarland tube No 0.5). The microtubes containing URE, LDC, ODC reagents were covered with 2 drops of mineral oil. After 4 hours of incubation at 37 C°. The reactions were read either using the mini API instrument or visually and recorded according to the standard results as indicated in the sheet shown in (Appendix - 3).

2-5 Effect of chemical and physical factors on the growth of *V.cholerae*:**2-5-1 Effect of NaCl:**

The effect of NaCl on the growth of *V.cholerae* was tested by the following method:

A . 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.

B . First tube was prepared by using same medium free from NaCl.

C . The tubes were inoculated with 0.1ml of freshly grown bacterial suspension and incubated for 20 hrs at 37C°, 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.

D . 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.

E . Spectrophotometer was also used to determine the optical density for each concentration to confirm the above experiment.

2-5-2 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} , and 10^{-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

2-5-3 Effect of sodium dichloroisocyanurate (NaDCC):

Different weight was used for obtaining four serial dilutions (25, 50, 75, and 100) mg/l of sodium dichloroisocyanurate in four test tubes. The pH was adjusted to 7.0 and each tube contains 9 ml of each concentration. Then all tubes were inoculated with 1.0 ml of bacterial suspension with turbidity equivalent to tube 1 McFarland solution which approximately equal to bacterial cells density of 3.0×10^8 cells /ml (MacFaddin, 2000).

All tubes were incubated at room temperature for 15, 30, 45, and 60 minute, then serial of tenth fold dilution (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6}) were prepared in sterile distal water for each concentration then the viable count of bacteria in each tube was estimated as in (2-5-2).

2-5-4 Effect of Garlic oil:

Plate of Müller-Hinton medium with 5 mm wells being previously; made by kork porer was prepared under aseptic condition. Three wells were made in an agar plate. *V.cholerae* suspension was made by adding growth from isolated colonies grown on a nutrient agar to 5 ml of nutrient broth and incubated for 2 hrs to produce bacterial suspension according to MacFaddin (2000).

A sterile cotton swab was used to obtain inoculums from *V.cholerae* suspension and streaked on the surface of Müller-Hinton medium. The two wells in Müller-Hinton medium were filled with the concentration of 0.25% and 0.5% of garlic oil extracted from garlic sativum. Another well was filled with normal saline as a control by using sterile micropipette under aseptic condition. The plate was incubated for 24 hrs at 37°C.

2-5-5 Effect of Temperature:

The effect of temperature on the growth of *V.cholerae* was experimentally determined by inoculating the isolated bacteria on different degrees of temperature ranged from 25°C up to 50°C by an increment of 5 degrees in alkaline peptone water medium. The inoculated tubes were incubated for 24 hrs then serial of tenth fold dilution 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} were used in sterile D.W for each test tubes to determine the viable count of bacteria as in (2-5-2).

2-6 Susceptibility to Antibiotics:

To detect the susceptibility of *V.cholerae* to a group of antibiotics, Kirby-Bauer method was used in which a pure culture of previously identified bacteria was prepared by adding growth from isolated colony to 5 ml of sterile normal saline in a cell density equivalent to turbidity of McFarland tube No. 0.5 which approximately equal to bacterial cells density of 1.5×10^8 cells/ml. A sterile cotton swab was used to obtain inoculum to be streaked on Müller-Hinton medium.

The antibiotic discs were placed on the surface of the medium at evenly spaced intervals with flaming sterile forceps. Incubation was usually overnight with optimal time of 18 hrs at 37°C. Antibiotic inhibition zone was measured using ruler. Zone diameter was compared to standard results being recommended by clinical laboratory standards institute documentations (CLSI, 2007).

2-7 Serological test:

2-7-1 Slide agglutination procedure:

Agglutination test for *V. cholerae* was carried out on a clean glass microscopic slide. Two drops of normal saline were dropped on a slide and a portion of *V. cholerae* growth from the surface of kligler iron agar was emulsified in few drops of sterile physiological normal saline and mix thoroughly using sterile applicator stick (Rahman *et al.*, 1989). Small drop of specific antiserum was added to the suspensions. Also two drops of normal saline was mixed with small drop of antiserum alone on the same slide without adding the growth of *V. cholerae* and this was used as negative control then the slide was tilted back and forth to observe for agglutination. The appearance of clumping (agglutination) within one minute referred to positive reaction compared to the homogenous mixture of the control. This test was used for serotype identification of *V. cholerae* O1 either Inaba or Ogawa serotype by using specific polyvalent and monovalent antisera specific for *V. cholerae* (Hasan *et al.*, 1994).

2-8 Preparation of antiserum:**2-8-1 Preparation of immunogen:**

For this experiment single *V.cholerae* isolate was used for preparation of specific immunogen. Somatic *V.cholerae* antigen was isolated and purified from bacterial cell wall that was killed by heat at 100 C°.

Nutrient agar was used for inoculated and growth of bacteria, after 18-20 hrs, the growth of bacteria was harvested by sterile loop in sterile screw-cap tube contains 10 ml sterile normal saline. After that bacterial suspension was placed in boiling water bath for 2½ hrs. At the end of heating period, the sterility of this suspension was checked by inoculating this suspension on nutrient agar and incubated for 24 hrs at 37 C°, and examined for growth. Suspension was stored in refrigerator then it was used for injected into laboratory animals as recommended by (Garvey *et al.*, 1981).

2-8-2 Intravenous injections (I.V) for rabbit:

Rabbit was placed in restraining position; topical anesthesia was used at site of injection using 2% lidocaine. Intravenous injection was performed in the marginal ear vein. The marginal vein is on the inner edge of the dorsal surface of the ear as in the figure (2-2). The base of ear pressed lightly and the blood that returns to the body was restricted in the vein which appears clearly. The injection area was cleaned with 70% alcohol then needle (26-gauge) was inserted into the vein. When the needle appears to be in the vein, if blood appears in the needle hub slowly the immunogenic content of the syringe were injected.

Sterile gauze was placed on the injection site as the needle is removed (Halliday *et al.*, 2004; Florida State University, 2007).

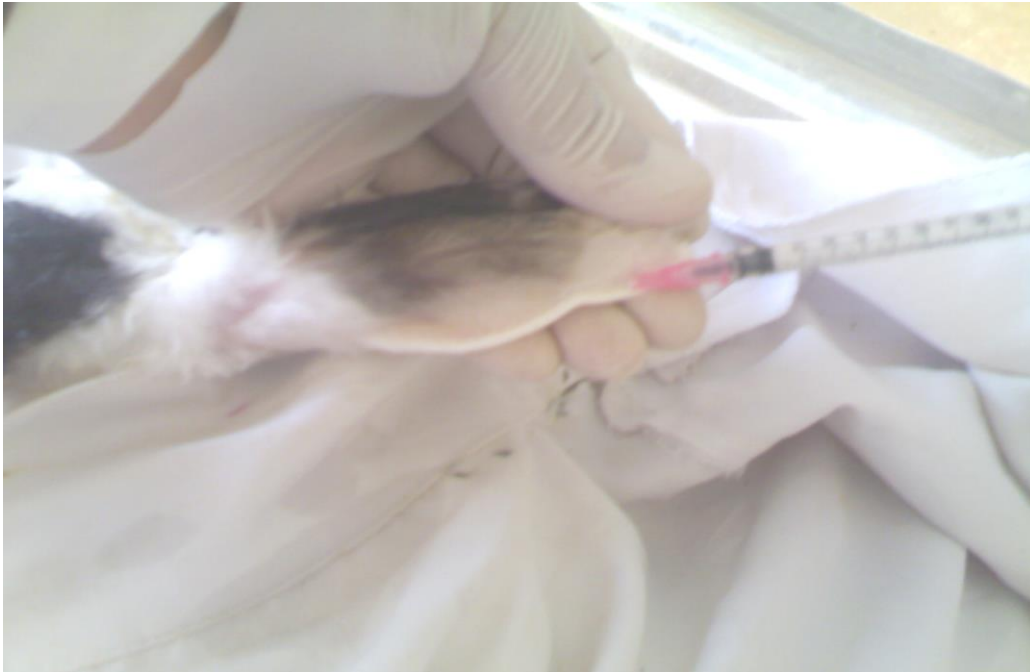


Figure (2-2):- Intravenous injection in the marginal ear vein of rabbit.

2-8-3 Intracardiac Blood Collection:

Intracardiac puncture for large volume blood collection was performed under general anesthesia using Diethyl ether 98%. The injection area was cleaned with 70% alcohol.

After that needle (22-gauge) was inserted into the lateral thoracic region toward the area of maximal heart beat between the ribs of the rabbit's left side midway between its sternum and back under the left elbow as shown in the figure (2-3). A volume of 10 ml of blood was collected slowly then sterile cotton was placed over the injection site as the needle is removed. The blood was decanted immediately in sterile test tube (Florida State University, 2007).

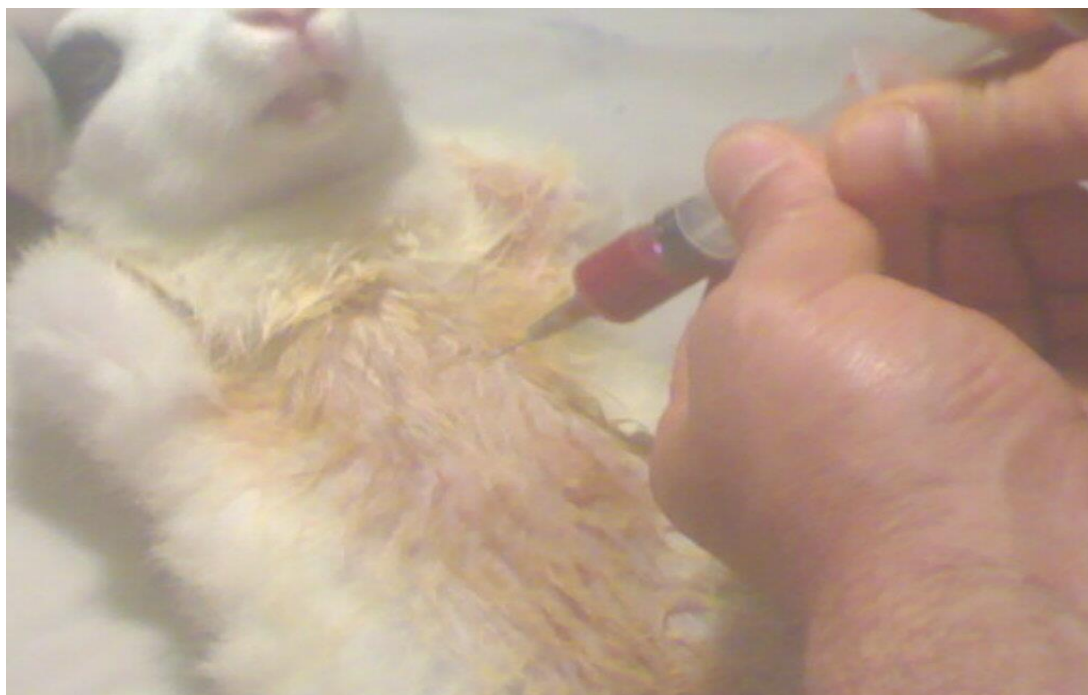


Figure (2-3):- Intracardiac blood collected from rabbit.

2-8-4 Separation and preservation of antisera:

Freshly blood that was collected from Rabbits allowed standing after collected for 2 hrs at room temperature for clot formation and centrifuged at (2500 rpm with a rotating radius of 14 cm for 10 minutes). Then the serum was removed carefully by sterile pipette and decanted into sterile Eppendrof test tube. Repeated centrifugation was performed by using Eppendrof centrifuge to obtain a clear serum.

Part of the serum was collected and stored in frozen at -18°C for long periods and the other part of serum was stored at (4°C) by addition of sodium azide at final concentration 0.10 % (Garvey *et al.*, 1981).

2-8-5 Titration of Antiserum:

Titration of antisera was performed using microtiter plate. Two fold method was used for obtaining serial dilution 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 by using normal physiological saline. All dilutions were tested with fresh growth of *V.cholerae* using slid agglutination test to determine the high dilution that give positive result. The titer was determined by multiply the reverse of high dilution give positive result by 10 (high dilution*10 = titer/ml) (Caponi and Migliorini, 1999).

2-9 Cross reaction test:

Cross reaction of prepared antisera was performed using slide agglutination test as in (2-7-1) with different types of bacteria (*Escherishia.coli*, *Salmonella typhi*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*) to determine the specific quality characteristics of prepared antisera (Garvey *et al.*, 1981).

2-10 Determination of Immunoglobulin Levels:**Single Radial Immunodifution (SRID) test:**

In this test, serum samples were added to wells in an agarous gel- containing a mono-specific antiserum. The sample diffuses radically through this gel and forms a precipitation ring with the mono-specific antiserum (Lowell, 2001). Precipitation ring diameters were measured by viewing device (ocular). The unknown concentrations were determined by comparing the ring

diameters with table supplemented with the kits (Lewis *et al.*, 2001).

Procedure

1. At the beginning the serums and the plates were equilibrated to room temperature.
2. Lids were removed from the plates. If moisture was present, plates were left uncovered to at room temperature (approximately 15minute) until moisture evaporated.
3. Serum samples were shaken and from each sample 5 μ l was dispensed into the wells.
4. The lid was replaced and the plates incubated at room temperature on level surface. Incubation times were 48 hours for IgG and 72 hours for IgM test.
5. Precipitation ring diameters were measured by viewing device (ocular) and compared to the standard diameter to calculate the concentration of total serum immunoglobulin (Lowell, 2001).

3-1 Bacteriological study:

3-1-1 Identification of *V.cholerae*:

In this study identification of *V.cholerae* depended on the colonial morphology, biochemical tests and serological tests.

The colonies of *V.cholerae* grown on cultures media represented by TCBS, MacConkey and blood agar at pH 9.0 and 37°C revealed the typical characteristics being described by referential studies, that the colonies were yellow shin color on the selective media TCBS agar due to fermentation of sucrose, with colonies diameter ranged from 2-3mm, these typical characteristics being described by referential studies (Sakazaki, 1992). The selectivity of TCBS is because it's containing NaCl and bile salt (Henry, 1996). In addition those colonies appeared as pale like shaped on the MacConkey agar indicated that *V.cholerae* was unable to ferment lactose sugar .On the blood agar it was a β -haemolytic and translucent colonies was appeared on the N.A. These results were in agreement with Forbes *et al.*, (2007).

Microscopical examination showed that *V. cholerae* was gram negative bacillus, comma shaped, non capsulated, not spore forming with specific darting movement using hanging drop method. More confirmation tests being recommended for identification of *V. cholerae* are shown in table (3-1). The results of these tests were in accordance with standard characters of this organism in the (Holt *et al.*, 1994; Collee *et al.*,1996; Forbes *et al.*, 2007)

Table (3-1):- The results of biochemical tests recommended for identification of *V.cholerae*O1.

The test	Results *
Gram stain	-
Growth on TCBS agar	+
Growth on MacConkey agar	+
Kligler iron agar	Alk /A No gas, No H ₂ S
Growth on semi solid media	Motile
Oxidase test	+
Catalase test	+
Indol test	+
String test	+
Urease test	-
Citrate test	+
Methyl red test	+
VP	+
Cholera red test	+
Starch utilizing	+
Gelatin liquefaction	+
ferment of Mannitol	+
ferment of Glucose	+
ferment of Sucrose	+
ferment of Arabinose	-

* + positive / - Negative

3-1-2 Results of API 20 E system:

This system is a standardized colorimetric identification system for enteric bacteria and it is considered as an acceptable method for identification of the more commonly-occurring members of the family *Vibrionaceae* (O'Hara *et al.*, 2003).

In this study API 20E system was used to confirm identification of *V. cholerae* included in this study. The results are shown in table (3-2) and (figure 3-1) and by using the analytical profile index of this system the identification percentage was (id% = 99.3) which was in compatible with Baron *et al.*, (2007), 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 *et al.*, (2001), 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.

In another study by Aidara *et al.*, (1998), who used API 20E system, serologic identification and susceptibility to antimicrobial agents to identify 117 isolates as *V.cholerae* O1 group from a total

Table (3-2):- Results of API 20E system used for identification of *V.cholerae*.

Test	Reactions/Enzymes	Results
ONPG	β -galactosidase Ortho NitroPhenyl - β D-Galactopyranosidase	+
ADH	Arginine DiHydrolase	-
LDC	Lysine DeCarboxylase	+
ODC	Ornithine DeCarboxylase	+
CIT	Citrate utilization	+
H ₂ S	H ₂ S production	-
URE	Urease	-
TDA	Tryptophane DeAminase	-
IND	Indole production	+
VP	Acetoin production (VogesProskauer)	+
GEL	Gelatinase	+
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	+

of 127 isolated obtained during the cholera epidemic in Senegal while 10 strains were identified as non-agglutinable *V. cholerae* (NAG).

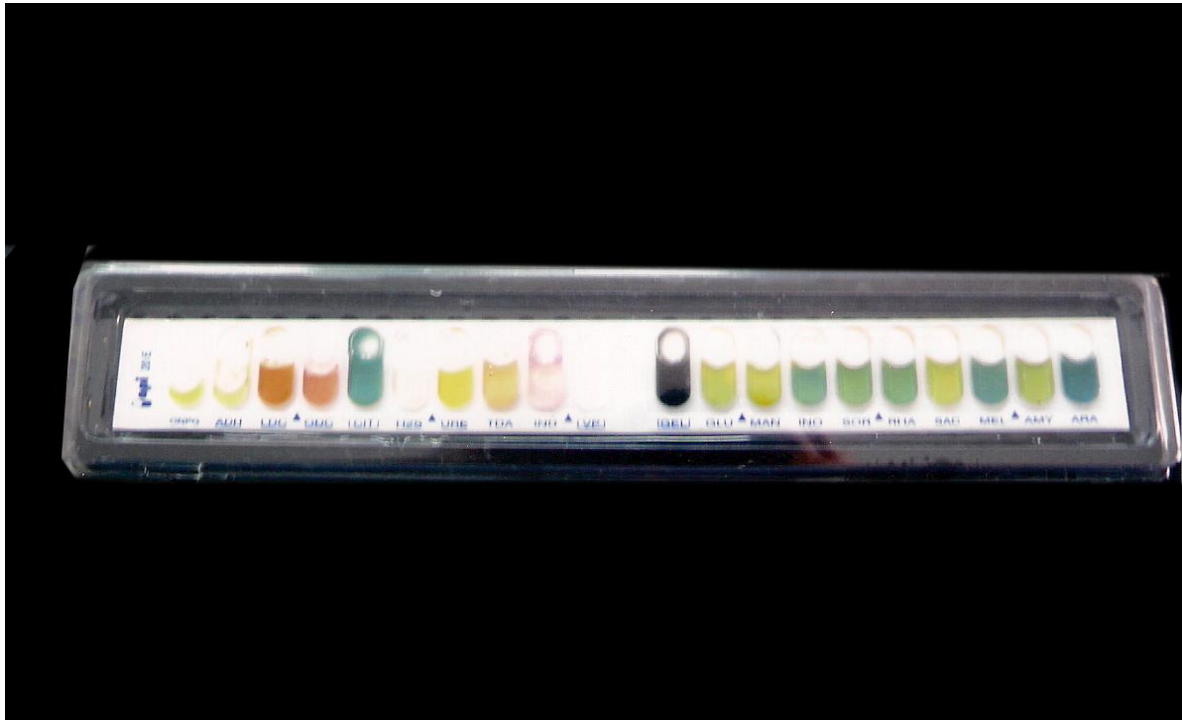


Figure (3-1) API 20E system used in this study

3-1-3 Result of Rapid ID 32E:

Identification of gram-negative bacilli, both enteric and non enteric by using of commercial kits, either manual or automated to identify these organisms is a common practice in today's world. This system was an upgraded version of the API 20E contains 32 substrates in a plastic-strip configuration and it provides more in-depth identification to the species level as well as detect new and

unusual strains (O'Hara, 2003). For this, Rapid ID 32 E (figure 3-2) was also used for identification of local *V. cholerae* isolate included in this study. This system can be read visually or Automatic Reading by using Mini Api device. The results of this study were shown in the appendix (1). It was used for the first time in Iraq in 2003 when the outbreak of cholera occurred and resulted in good and rapid results within four hrs for identification of *V.cholerae* Al-Karkhy (2003).

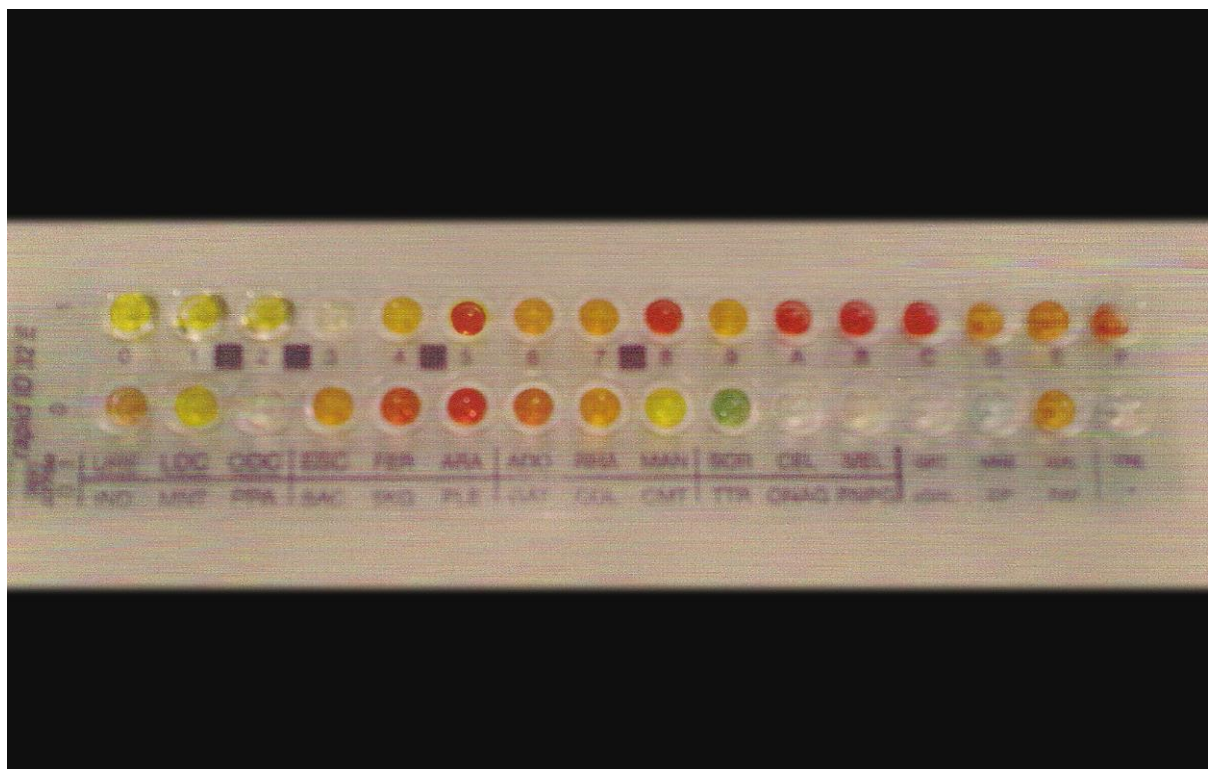


Figure (3-2) Rapid ID 32E system used in this study

3-2 Investigation of some factors associated with the pathogenicity of *V.cholerae*.

3-2-1 Detection of motility of *V.cholerae*:

Semisolid medium and hanging drop method were used to detect the motility of *V.cholerae* in this study, and the results of these tests showed that this organism is motile, giving diffuse hazy growth in semisolid medium and what it is always described as darting movement in hanging drop method (Finklestein *et al.*, 1992). This motility is seemed to be closely related with pathogenicity, since the surviving bacteria exit the stomach and reach the small intestine they need to propel themselves through the thick mucus that lines the small intestine to get to the intestinal wall where they can thrive (Todar, 2008). Freter *et al.*, (1981) have shown that motile *V. cholerae* organisms direct themselves to the mucosal surface in response to chemotaxins.

V.cholerae produces the hollow cylindrical protein (flagellin) to make flagella, the curly whip-like tails that they rotate to propel themselves through the mucus that lines the small intestine (Hartwell *et al.*, 2004). A study by Richardson (1991) concluded that motility was clearly a major contributing factor to *V. cholerae* pathogenicity and colonization while the flagellar structure alone seemed to be less important.

3-2-2 Detection of 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 is able to produce the haemolysin. The appearance of a clearly halo 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 (Neilands, 1995). 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* (Brown and Manning, 1995). Ichinose *et al*, (1987) 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.

3-2-3 Detection of protease enzyme:

In this study *V.cholerae* O1 Inaba was tested for its ability to produce protease enzyme. The results showed that *V.cholerae* has the ability to hydrolyze the protein by protease enzyme when inoculated on skim milk agar and incubated for 24 hours at 37°C.

A halo zone appeared around the wells as shown in figure (3-3). Many studies indicated that in order to cause disease, *V. cholerae* needs to express numerous virulence factors in response to host signals (Atsumi *et al.*, 1996). Brown & Häse (2001) observed that the production of extracellular proteases could allow *V. cholerae* swimming by decreasing the viscosity of the medium, which helps in speeding of this organism. *Vibrio cholerae* produces a soluble Zn^{2+} -dependent metalloprotease (mucinase) this enzyme encoded by *hapA* gene (Miyoshi & Shinoda, 2000) and can activate cholera toxin A subunit and hydrolyse several physiologically important proteins such as mucine, fibronectine (Silva *et al.*, 2003).



Figure (3-3) Protease enzyme production on skim milk agar

3-3 Effect of NaCl on the growth of *V. cholerae*:

The results obtained from this study showed that 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 3% NaCl concentration but rate of the growth decreased with an increasing of NaCl concentration to reach the minimum growth at 6% of NaCl concentration. At 6.5% NaCl no growth was seen as shown in the figure (3-4). These results were in compatible with Pflughoeft *et al.*, (2003), who pointed out that there is a correlation between relative increased salinity and the incidence of cholera in the Indian subcontinent and found that *V.cholerae* was reached to optimum growth with medium supplemented with 2.8% NaCl (Dunlap, 2009). Moreover the results were in agreement with results obtained by Kolvin and Roberts, (1982) who indicated that growth of *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).

Recently, three genes *nhaA*, *nhaB* and *nhaD* have been identified in *V. cholerae*. These genes are presumably involved in the survival and persistence of free-living bacteria in their natural habitats and responsible for the survival of *V. cholerae* and their colonization in intestine in response to high salt concentration (Vimot and Berche, 2000; Dzioba *et al.*, 2002).

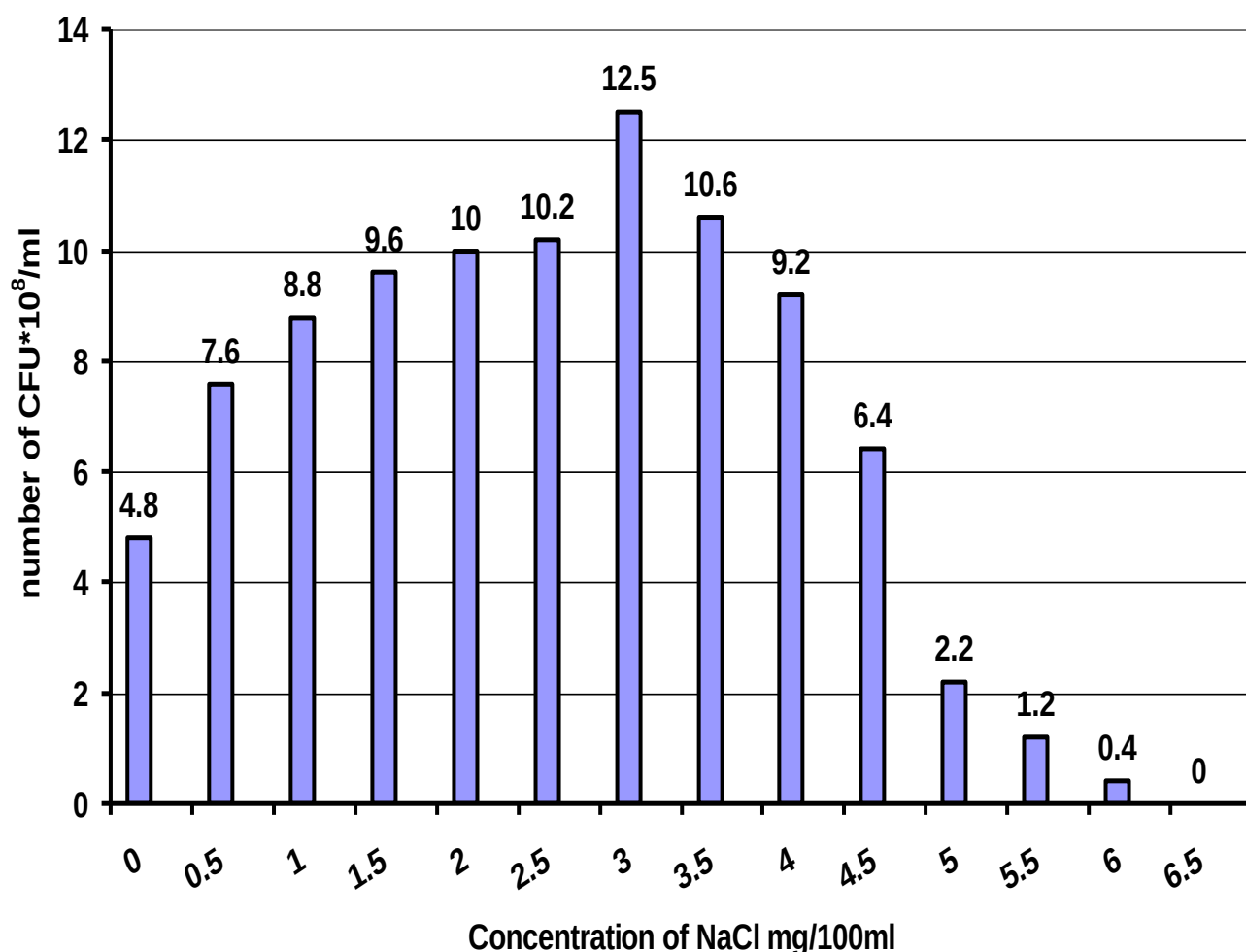


Figure (3-4):- Effect of the NaCl on the growth of *V.cholerae* for 20 hrs, at 37°C

3-4 Effect of pH on the growth of *V. cholerae*:

The results of this study revealed that there was a tolerant of this local isolate to alkaline condition which reaches up to pH 10.0 and the growth began to decline under pH 8.0 and above 9.5, while there was no growth under pH 5.5 and above pH 10.5.

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. These results are agreeable with those results obtained by Patel and Isaacson (1999), who confirmed that *V.cholerae* O1 grow well at pH 9.0 and in another 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.

In addition, Vibrios are sensitive to low pH and die rapidly in solution below pH 6.0; 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).

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 (2003) 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).

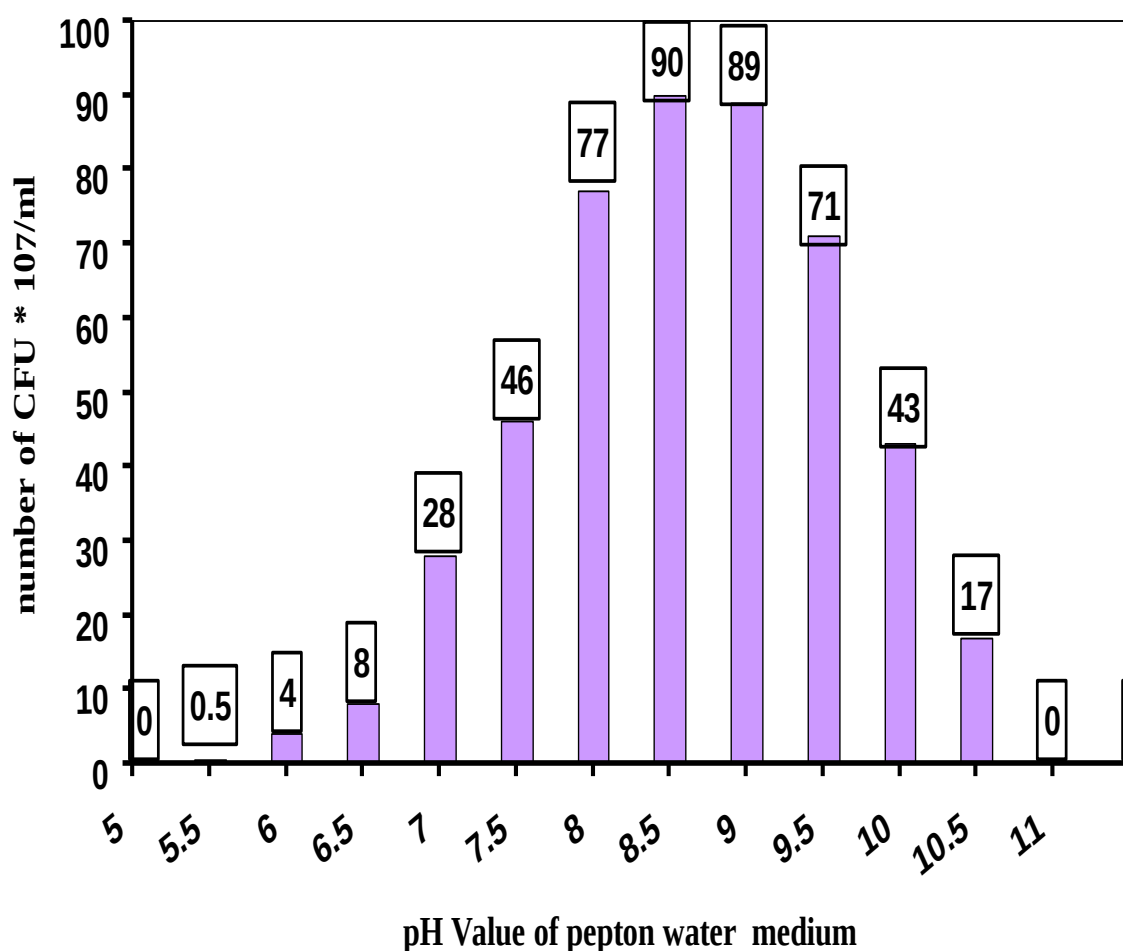


Figure (3-5):- Effect of pH on the growth of *V.cholerae* at 37°C for 20 hrs,

3-5 Effect of sodium dichloroisocyanurate (NaDCC) on the growth of *V. cholerae*:

Different concentrations of NaDCC were used with different periods as shown in the figure (3-6) to detect the efficacy of this compound on viability of *V.cholerae* when it is used as disinfectant of drinking-water.

The results showed that there were proportionally increased in killing of *V.cholerae* with increased concentration of NaDCC. *V.cholerae* were totally killed by 5.0 mg/L and 7.5 mg/L of NaDCC within 60 min, while it killed within 45 min in a

concentration of 10.0 mg/L, even though some disadvantages may appear at this concentration such as taste of treated water which become unacceptable. However, These findings were also compatible with results recommended by WHO, (2003) which reported the guideline value was 5 mg/L for free active chlorine (FAC) and development of 1 mg of FAC per liter typical for drinking-water treatment requires approximately 1.6 mg of NaDCC per liter and should not normally exceed this value.

Sodium dichloroisocyanurate is the sodium salt of a chlorinated hydroxytriazine used as a source of Free Available Chlorine (FAC) in the form of hypochlorous acid, for the disinfection of water. It was widely used as a stable source of chlorine for the disinfection of swimming pools and in the food industry, since it is more stable in sun light than most other sources of chlorine. It was also used as a means of disinfecting drinking-water, primarily in emergencies, when it provides an easy-to-use source of free chlorine (Pinto & Rohrig, 2003). As long as NaDCC is added to water to maintain a certain level of FAC, and the level depending on the properties of the water such as pH and temperature (Kuznesof, 2003).

According to our observation in AL-Ghazally village this village was the focus of cholera in Babylon province. NaDCC if used; the treated water was used immediately after the addition of NaDCC tablet.

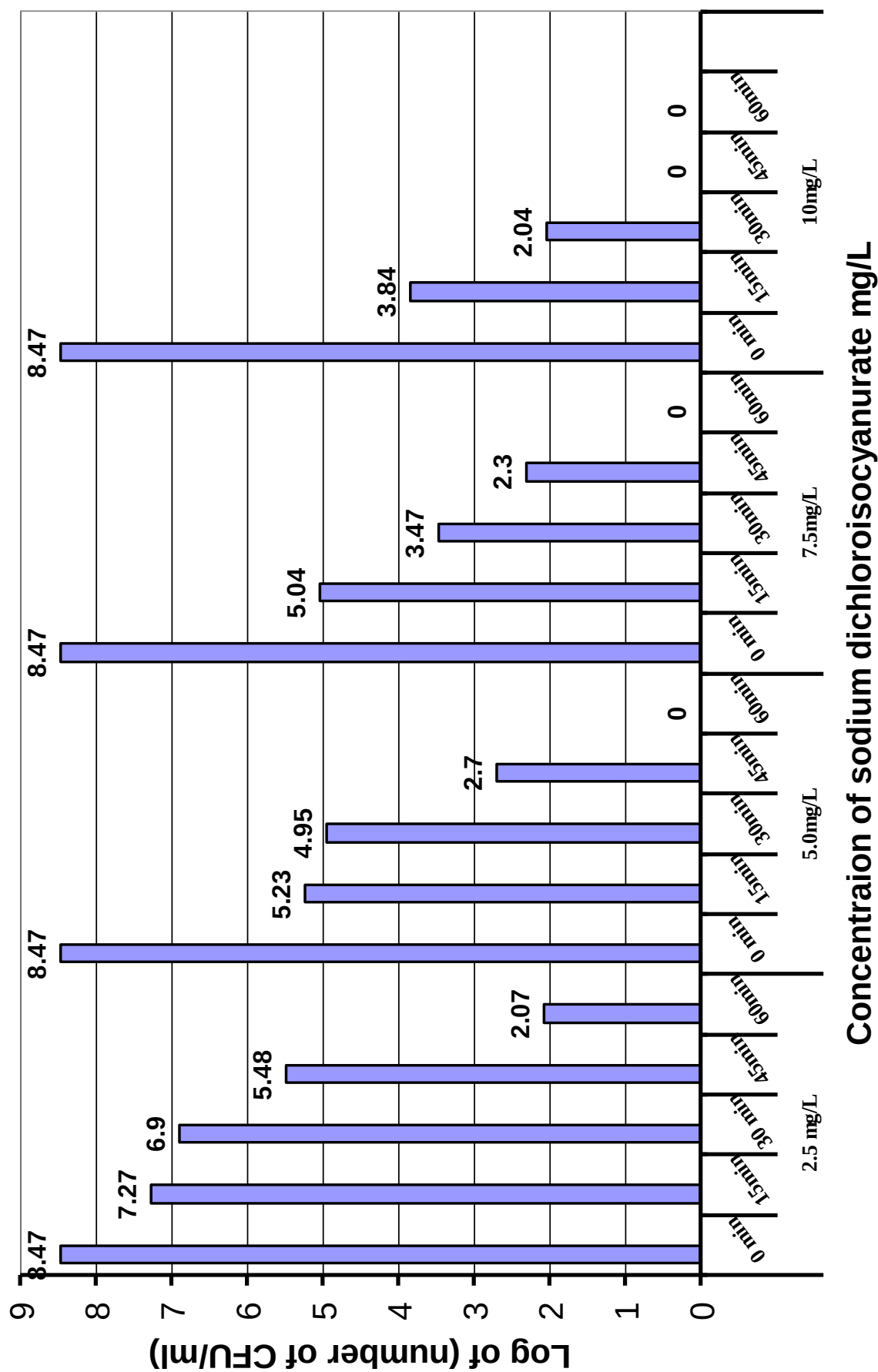


Figure (3-6):- Effect of sodium dichloroisocyanurate (NaDCC) on the growth of *V. cholerae*.

3-6 Effect of Garlic oil on the growth of *V. cholerae*:

In this study the effect of garlic oil on the growth of *V. cholerae* isolated in Babylon province through the outbreak of cholerae disease was examined.

The results of this experiment using garlic oil 0.25% and 0.5% showed that there was no effect of this plant extract on the growth of *V. cholerae* indicated by no inhibition zone around the wells compared with control well, filled with normal saline.

The antibacterial effect of garlic apparently results from interaction of sulphur compounds, like allicin, with sulphur (thiol) groups of microbial enzymes (trypsin and other proteases), leading to an inhibition of microbial growth (Jonkers *et al.*, 1999; Bakri and Douglas, 2005). Allicin; which is a Thio sulphate compound was thought to confer many of garlic's medicinal effects, but garlic oil was also shown to be metabolized to a number of additional organosulphur compounds (Khanum *et al.*, 2004).

In other study by Pan American Health Organization (1991) showed that spices, including raw onions and garlic can support the survival of *V. cholerae* for 2 to 3 days at ambient temperature. Lai and Roy (2004) were mentioned that the antibacterial properties of garlic can be eliminated by some organisms by inhibition of the allinase enzyme and prevention of allicin formation.

3-7 Effect of Temperature on the growth of *V.cholerae*:

The effect of temperature on the growth of local isolate of *V.cholerae* was examined in this study.

Figure (3-7) shows the mean levels growth of *V.cholerae* in different degrees of temperature ranged from 25°C to 50°C. The results revealed that high values of growth were observed between 30°C to 35°C and the optimum temperature was 35 °C, since the cell density was 13.6×10^8 CFU/ml while there was no growth at 50°C. Also the result showed that *V.cholerae* exhibited a degree of tolerance at 45°C 1×10^8 CFU/ml and the colonies were appeared smaller at 25°C when compared to other temperature degrees.

These results were in accordance with those results obtained by Richards and Watson (2006) who stated that *V.cholerae* could be incubated at temperatures ranging between 22°C to 37°C but the colonies were smaller when incubated at the lower temperatures.

Another study by Borroto and Piedra (2000) indicated that the optimal temperature for *V. cholerae* O1 multiplication was 30–37°C and the growth was inhibited below 20°C. The growth of *V. cholerae* O1 was summarized by ICMSF (1996) which observed that the optimum temperature for growth is 37°C with a range of (20 to 43)°C .

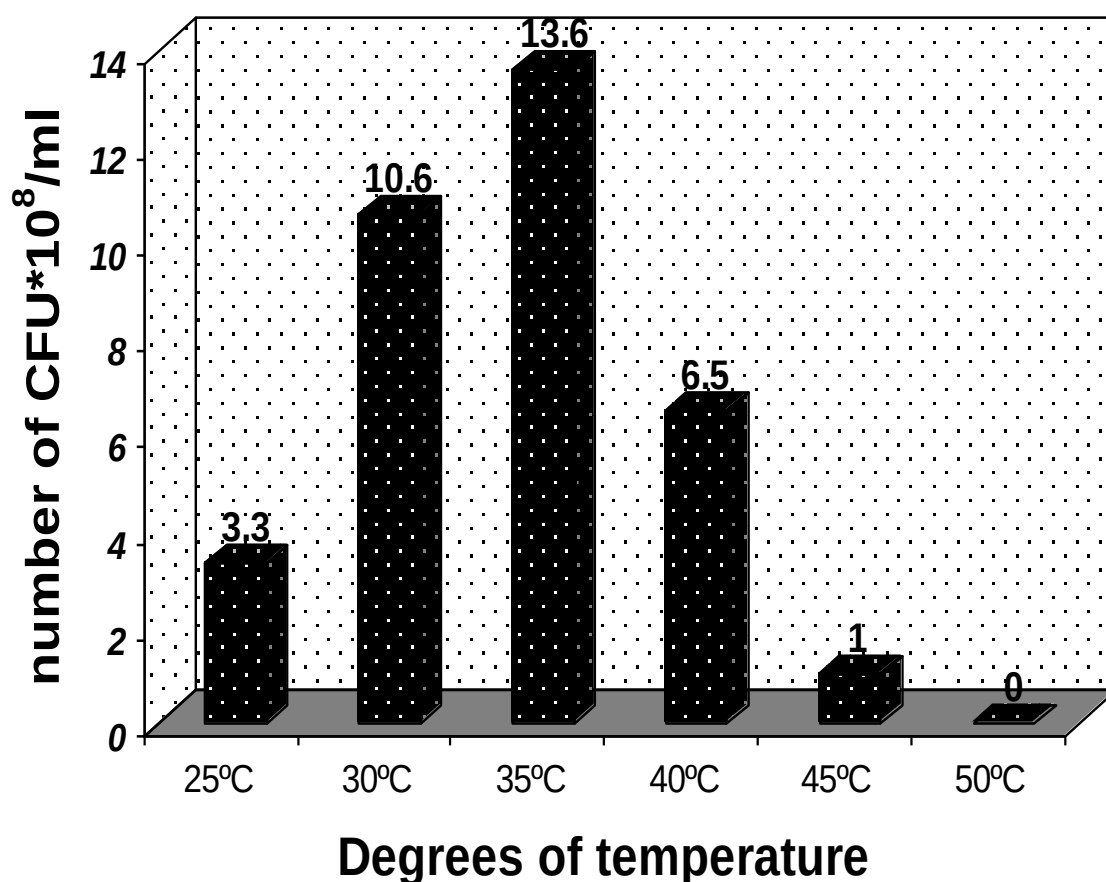


Figure (3-7):- The effect of temperature on the growth of *V.cholerae*

3-8 Effect of Antibiotics on *V.cholerae*:

Seven antibiotics were used in this study to determine their effects on the local isolate of *V.cholerae* O1 Inaba. The results indicted that *V.cholerae* exhibited a clear sensitivity towards Tetracycline, Doxycycline, Erythromycin and Chloramphenicol and resistance to Amoxicillin, Ciprofloxacin and trimethoprim-sulfamethoxazole.

The results exhibited that local isolate of *V.cholerae* was sensitive to Tetracycline and Doxycycline with an inhibition zone 20 mm and 19 mm respectively as shown in table (3-4).

Rabbani and Greenough (1999) observed that treatment with an effective antimicrobial agent reduced fluid loss and the duration of diarrhoea, but this form of treatment is not considered a substitute for rehydration therapy. Since untreated diarrhoeal stools from cholera patients are the primary source of environmental contamination, including water sources and foods, proper treatment of cholera cases and safe disposal of faeces would reduce secondary spread and faecal contamination of the environment.

A recent study by Khan *et al.*, (2003) who found in total, 157 patients were enrolled into the study and all were infected with the El Tor biotype of *V. cholerae* O1 with either Inaba or Ogawa serotype. Isolates of 130 patients were sensitive to tetracycline. Alam and co-workers (2006) used a single dose of Doxycycline (5 mg/kg) for treatment of children infected with *V.cholerae*. They reported that 90% of these cases responded to that dose of Doxycycline. However, in children, it is well known that the adverse effects like enamel and skeletal defects associated with tetracycline occurred on prolonged use of tetracycline (Kaper *et al.*, 1995).

Widespread use of tetracycline prophylaxis was associated with rapid development of antimicrobial resistance and should be strongly discouraged (Kaper *et al.*, 1995).

The isolate of *V. cholerae* in this study was found to be susceptible to Chloramphenicol and revealed intermediate inhibition zone about 15mm when it was compared according to the (CLSI, 2007)

This result was in agreement with the result obtained by Gotuzzo *et al.*, (199) who reported that can be used of Chloramphenicol in treatment of adult cholera patients. However, variable susceptibility of *V.cholerae* toward Chloramphenicol has been reported by some studies. Al-Karkhy (2003) in Iraq indicated a highly sensitivity of local isolate to Chloramphenicol, which in accordance with the results of the study. While Amin *et al.*, (1995) in India indicated that *V.cholerae* was highly resistance to Chloramphenicol this difference in antibiogram susceptibility to *V. cholerae* may be due to different biotypes.

In addition to that, the locally isolate of *V.cholerae* showed resistant to ciprofloxacin as shown in the table (3-4) and this result disagrees with a study by Alam *et al.*,(2006) who found a successful treatment in 60% cases isolated in India with a single dose ciprofloxacin 20 mg/kg. Ciprofloxacin is a bacteriocidal drug and affected on both Gram positive and negative bacteria. The resistance to antibiotic was through alteration affecting the ability of antibiotic to penetrate bacterial cell walls (Weld and Sandham ,1999).

Also, the results showed that *V.cholerae* O1 Inaba was resistant to the Vibriostatic compound O/129 (2,4-diamino-6,7diisopropyl pteridine) and trimethoprim-sulfamethoxazole.

These results were similar to results of Nath and Sanyal (1992) who reported that resistance to O/129 usually associated with resistance to trimethoprim-sulfamethoxazole. In addition, another study by Al-Karkhy (2003) who found a high level of resistance 100% of *V.cholerae* O1 Inaba and Ogawa to trimethoprim-sulfamethoxazole and to the Vibriostatic compound O/129.

Amoxicillin was also examined and the result showed there was resistance of *V.cholerae* to this antibiotic. The results of this study are compatible to a large extent with results obtained by Aidara *et al.*, (1998) in Guinea-Bissau and Ryzhko *et al.*, (2005) in India who found that *V. cholerae* El Tor O1 strain isolated from a cholera patients showed highly resistant to amoxicillin, ampicilin , trimethoprim/sulfamethoxazole and ciprofloxacin and revealed that the only efficient antibiotics were tetracycline.

Erythromycin was examined in this study and the result showed sensitivity of *V.cholerae* to this antibiotic. This result was in agreement with the result obtained by Keramat and his worker (2008) who found a successful treatment with Erythromycin in 73 cases of 100 specimens of *V. cholerae* isolated in Iran and 27 showed resistances to this antibiotic. Erythromycin can be used in the treatment of children less than 7 years and pregnant women (Seas *et al.*, 2005).

Table (3-3) Effect of Antibiotics on the growth of *V.cholerae*.

Antimicrobial agent	Disk potency (µg)	Zone Diameter in mm (CLSI*)			The results (mm)
		R*	I*	S*	
Amoxicillin	25	≤ 13	14-16	≥ 17	11-(R)
Tetracycline	30	≤ 14	15-18	≥ 19	22-(S)
Doxycycline	30	≤ 14	15-18	≥ 19	19-(S)
Chloramphenicol	30	≤12	13-17	≥ 18	15-(I)
Erythromycin	15	≤ 13	14-16	≥ 17	18-(S)
Ciprofloxacin	5	≤15	16-20	≥ 21	(R)
Trimethoprim-sulfamethoxazole	1.25+ 23.57	≤10	11-15	≥ 16	No effect (R)
Vibriostatic disc (O/129)	150				No effect (R)

*CLSI-Clinical and Laboratory Standard Institute.

*R-Resistance.

*I-Intermediate.

*S-Sensitive.

3-9 Preparation of antisera:

In this study, local diagnostic antisera was prepared by using somatic antigen of *V.cholerae* serotype O1 Inaba strain, since antisera were widely used to rapidly confirm the diagnosis of *V.cholerae* and to differentiate the serotypes of this organism (Colwell *et al.*, 1992; Qadri *et al.*, 1994). NewZeeland albino female rabbits were used to prepare the local diagnostic antisera. These animals are cost-benefit ratio, ease of handling and hyper immune which respond rapidly when injected with antigens as recommended by (Florida State University, 2007).

3-10 Titration of antisra:

In this study titration of prepared antisera which was obtained from laboratory animals was performed to determine the potency and efficiency of these antisera in relative to other available antisera. The results as shown in the figure (3-8) revealed that antisera obtained from rabbit A gave 2560 unit/ml while those obtained from rabbit B gave 1280 unit/ml. These results were high in comparison with commercial foreign antisera (Mast, England) which gave titer 80unit/ml.

This result was in agreement with Barua and Burrows (1974) who referred that sera obtained from lab animals (rabbits) usually has an agglutinin titer of 2650 to 5120 unit/ml when tested with live *V.cholerae* antigen. The high titer increases the diagnostic value of serologic test used in the identification of infectious agents at the same time decreases the chance for the possibility of cross-reacting (Rich *et al.*, 2009).

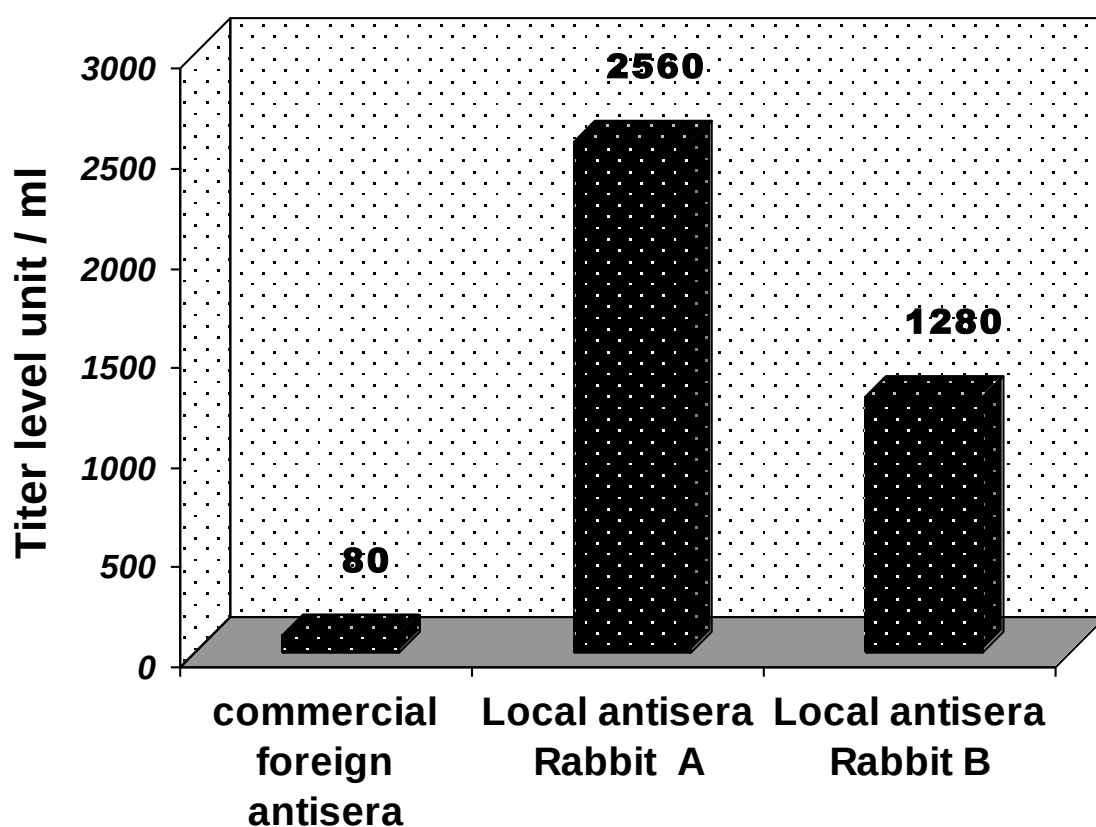


Figure (3-8):- Titration of Antisera.

3-11The concentration of IgG and IgM:

In this study single radial immunodiffusion (SRID) method was used to determine the profile of humoral, B cell response; of laboratory animals to the somatic antigen as shown in the figure (3-9) and (3-10). The results are expressed in table (3-5) which shows that there was high in the serum level of IgM of laboratory animals (3000 mg/L) for rabbit A and (2400 mg/L) for the rabbit B in comparison with control animal being not injected with somatic antigen which gave (859 mg/L). Regarding the total

serum levels of IgG as shown in table (3-5) shows that there is no high difference(only 300mg/l) between rabbit A and rabbit B but both of them are high when compared with the total serum level of IgG of control animal which gave 24500mg/L.

The clear differences in the IgM concentrations of immunized and control rabbits may indicate the primary and acute infection or artificially induced infection as carried out in this work depending on the facts that IgM level elevates during the primary infection (Rich *et al.*, 2003; Tizard, 2009). This indicates that local prepared cholera immunogen can induce a well- characterized immunity represented by hyper immune response.

Table (3-5):- Concentration of total IgM & IgG (mg/L) in cholerae immunized and control rabbits.

sample	IgM mg/L	IgG mg/L
Rabbit (A)	3000	36700
Rabbit (B)	2400	37000
Control (C)	859	24500



Figure (3-9):- The single radial immunodiffusion (SRID) assay for IgM concentration



Figure (3-10):- The single radial immunodiffusion (SRID) assay for IgG concentration

3-12 Cross reaction test:

Cross reaction was performed in this study to determine the specificity characteristics of prepared antisera. It was showed that there were negative results with all tested bacteria used in this test. These results indicted to high specific quality characteristics of this antisera and can be used it in diagnosis of *V.cholerae*.

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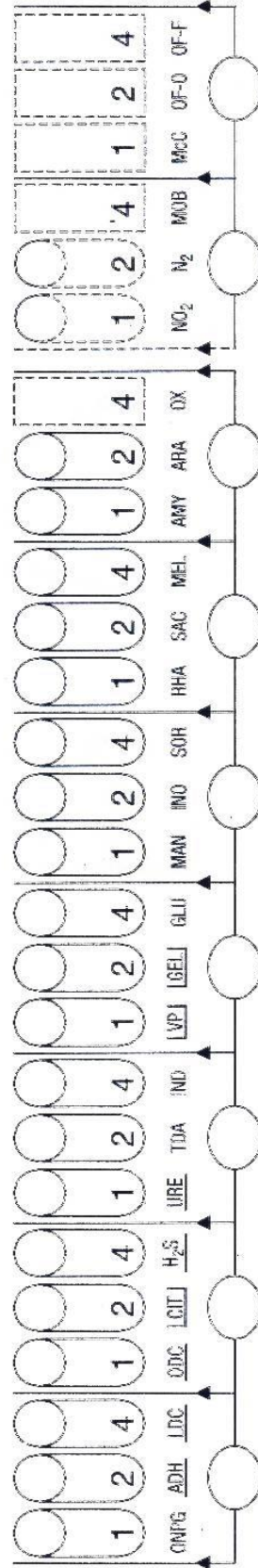
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Appendix (1):- Results of Rapid ID 32E for *V.cholerae*.

Test	Reactions/Enzymes	Results
URE	Urease	-
LDC	Lysine DeCarboxylase	+
ODC	Ornithine DeCarboxylase	+
ESC	Esculin	-
FER	Fermentation	+
ARA	Arabinose	-
ADO	Adonitol	-
RHA	Rhamnose	-
MAN	Mannitol	+
SOR	Sorbitol	-
CEL	Celiobiose	-
MEL	Melibiose	-
GRT	Glucuronate	-
MNE	Mannose	+
MAL	Maltose	+
TRE	Trehalose	+
IND	Indole	+
MNT	Malonate	-
PPA	ParaPhenylAlanine deaminase	-
SAC	SaCcharose	+
5KG	5 KetoGluconate	-
PLE	Palatinose	-
GAT	Galactouronate	-
COL	Colistin	+
CMT	Coumorate	+
TTR	Tetra Thionate Reductase	-
ONAG	O-Nitrophnyl N-Acetyl- β D-Glucosaminide	+
PNPG	P-Nitrophenyl- β D-Galactopyranoside	+
α GAL	α Galactosidase	-
IDP	Indoxyl Phosphate	-
RAF	Rafinose	-
OX	Oxidase test	+

api® 20E

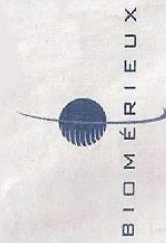


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Appendix (2) Reaction reading table of API-20 E system

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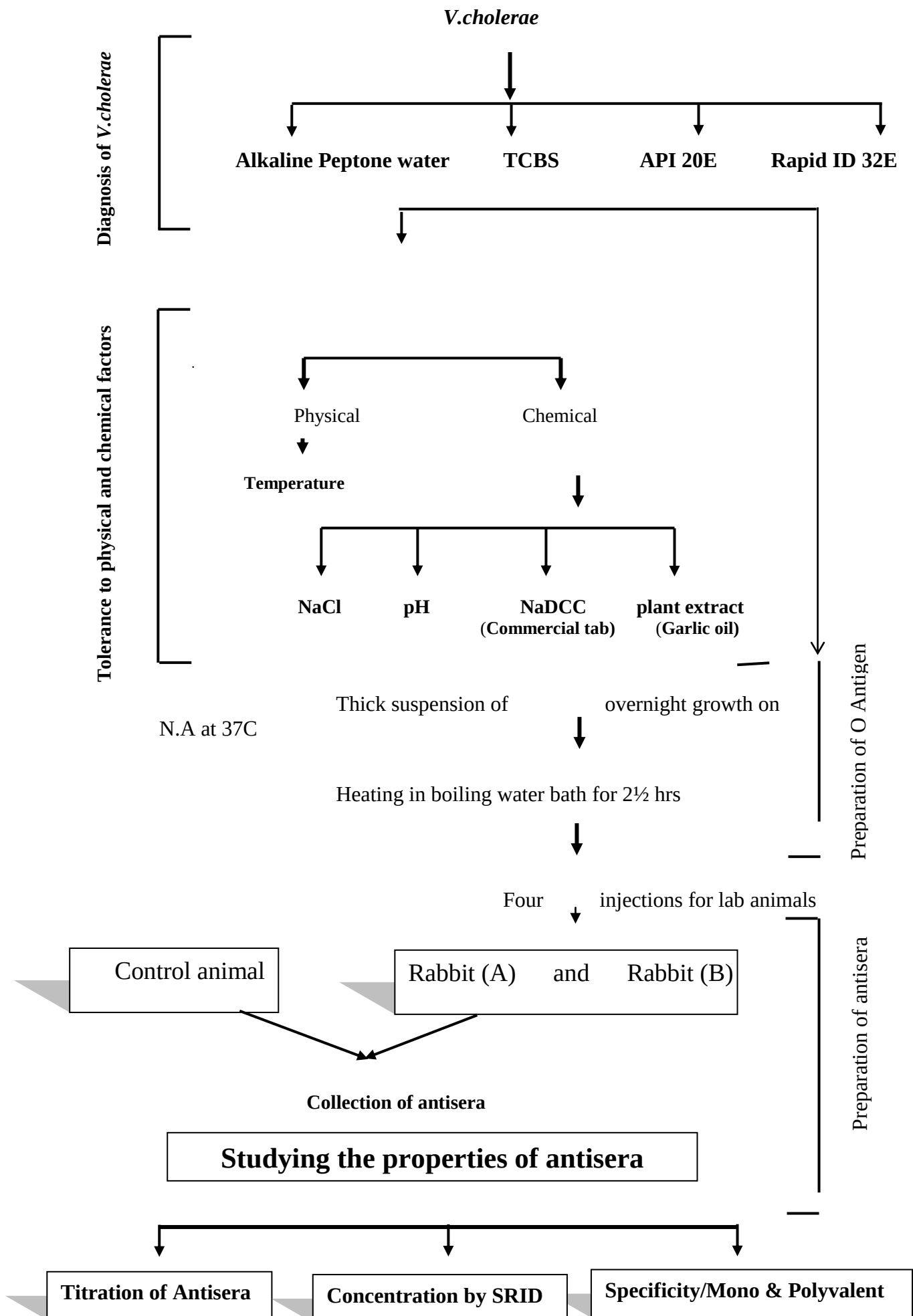
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URE	LDC	QDC	ESC	FER	ARA	PLE	GAT	ADO	RHA	MAN	SOR	CEL	MEL	URE	LDC	QDC	ESC	FER	ARA	PLE	GAT	ADO	RHA	MAN	SOR	CEL	MEL	URE	LDC	QDC	ESC	FER	ARA	PLE	GAT	ADO	RHA	MAN	SOR	CEL	MEL	URE	LDC	QDC	ESC	FER	ARA	PLE	GAT	ADO	RHA	MAN	SOR	CEL	MEL	URE	LDC	QDC	ESC	FER	ARA	PLE	GAT	ADO	RHA	MAN	SOR	CEL	MEL	URE	LDC	QDC	ESC	FER	ARA	PLE	GAT	ADO	RHA	MAN	SOR	CEL	MEL						
IND	MNT	PPA	SAC	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	IND	MNT	PPA	SAC	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	IND	MNT	PPA	SAC	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	IND	MNT	PPA	SAC	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	IND	MNT	PPA	SAC	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	IND	MNT	PPA	SAC	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG	5KG						
1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
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Appendix (3) Reaction reading table of Rapid ID 32E system

Index (1):- Results of Rapid ID 32E for *V.cholerae*.

Test	Reactions/Enzymes	Results
URE	Urease	-
LDC	Lysine DeCarboxylase	+
ODC	Ornithine DeCarboxylase	+
ESC	Esculin	-
FER	Fermentation	+
ARA	Arabinose	-
ADO	Adonitol	-
RHA	Rhamnose	-
MAN	Mannitol	+
SOR	Sorbitol	-
CEL	Celiobiose	-
MEL	Melibiose	-
GRT	Glucuronate	-
MNE	Mannose	+
MAL	Maltose	+
TRE	Trehalose	+
IND	Indole	+
MNT	Malonate	-
PPA	ParaPhenylAlanine deaminase	-
SAC	SaCcharose	+
5KG	5 KetoGluconate	-
PLE	Palatinose	-
GAT	Galactouronate	-
COL	Colistin	+
CMT	Coumorate	+
TTR	Tetra Thionate Reductase	-
ONAG	O-Nitrophnyl N-Acetyl- β D-Glucosaminide	+
PNPG	P-Nitrophenyl- β D-Galactopyranoside	+
α GAL	α Galactosidase	-
IDP	Indoxyl Phosphate	-
RAF	Rafinose	-
OX	Oxidase test	+



Appendix (1) Schematic diagram illustrates the main steps of the work

Table (3-3) : Effect of sodium dichloroisocyanurate (NaDCC) on the growth of *V. cholerae*

Concentration of sodium dichloroisocyanurate	Time in minute	Log of (number of CFU/ml)
2.5 mg /L	0 min	8.47
	15 min	7.27
	30 min	6.9
	45 min	5.48
	60 min	2.07
5.0 mg /L	0 min	8.47
	15 min	5.23
	30 min	4.95
	45 min	2.7
	60 min	0
7.5 mg/L	0 min	8.47
	15 min	5.04
	30 min	3.47
	45 min	2.3
	60 min	0
10 mg /L	0 min	8.47
	15 min	3.84
	30 min	2.04
	45 min	0

	60 min	0
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الخلاصة

أجريت هذه الدراسة على إحدى عزلات ضمات الهيضة *Vibrio.cholerae* تم عزلها من إحدى الحالات السريرية (الأسهال المائي) في مستشفى الحلة التعليمي خلال شهر أيلول 2008 . تضمنت الدراسة ثلاثة محاور رئيسية , المحور الأول بكتريولوجي وتضمن دراسة تشخيصية للعزلة وفق أنظمة تشخيصية متقدمة ومعتمدة وتضمن المحور الثاني الكشف عن بعض عوامل الضراوة التي تمتلكها البكتريا وقدرة البكتريا على تحمل بعض العوامل الفيزيائية والكيميائية, أما المحور الثالث فقد تضمن دراسة مناعية تم من خلالها تحضير المصل التشخيصي متعدد التكافؤ Polyvalent antisera وقياس مستوى الكلوبينات المناعية نوع IgM و IgG بطريقة الانتشار المناعي الشعاعي المفرد.

عزلة واحدة لبكتريا الكوليرا أستخدمت في هذه الدراسة. تم اختبار البكتريا من خلال أجراء الفحوصات المجهرية والكيموحياتية ومن ثم تم تشخيصها بأستخدام نظام API 20E ونظام Rapid ID 32E ثم التشخيص المصلي لتحديد النمط المصلي للبكتريا. ومن خلال نتائج تلك الفحوصات ومقارنتها بالصفات النوعية للبكتريا تأكد بأنه العزلة قيد الدراسة هي *V.cholerae* النمط المصلي إنابا. أظهرت النتائج قدرة البكتريا على النمو والتكاثر في مديات واسعة نسبيا لدرجات الحرارة والأس الهيدروجيني تراوحت من 25°م الى 45°م وان الدرجة الفضلى هي 35°م وانها لا تتمكن من النمو بدرجة حرارة اعلى من 45°م . اما مستوى الاس الهيدروجيني الذي تمكنت البكتريا من النمو فيه فقد تراوح من 5.5 الى 10.5 ولا تستطيع النمو تحت او فوق هذين المستويين غير ان افضل مدى هو pH 8.5.

تمكنت البكتريا قيد الدراسة من النمو في تراكيز مختلفة لكوريد الصوديوم وأظهرت النتائج أن كلوريد الصوديوم يحفز البكتريا على النمو حيث كان التركيز الأفضل هو 3% بدأ النمو بعده بالتناقص وصولاً إلى 6.5% . كذلك اظهرت البكتريا مقاومة شديدة لزيت الثوم. اظهرت النتائج ان افضل تركيز لمادة (NaDCC) Sodium dichloroisocyanurate المستخدمة في تطهير ماء الشرب بشكل آمن

هو 5مج/ل لمدة 60 دقيقة في درجة حرارة الغرفة مع الحفاظ نسبيا على صفات الماء النوعية من طعم و رائحة.

كشفت الدراسة عن امتلاك بكتريا الكوليرا لبعض عوامل الضراوة متمثلة بـأنتاج محلل الدم نوع (β) و الانزيم المحلل للبروتينات protease enzyme اضافة الى قدرة البكتريا على الحركة بنشاط مما يمكنها من اختراق الطبقة المخاطية المبطنة للامعاء.

ومن النتائج التي توصلت اليها الدراسة قدرة البكتريا على مقاومة المضادات الحيوية الاموكسيلين والترايمثوبريم مع السلفاميثاكسازول وكذلك السيبروفلوكساسين غير انها حساسة للمضادات الحيوية التتراسايكلين, والدوكسي سايكلين , والارثرومايسين, و الكلورمفينيكول. و أظهر المضاد التتراسايكلين فعالية عالية في قتل الجرثومة تجريبيا.

المصل التشخيصي متعدد التكافؤ Polyvalent diagnostic antisera تم تحضيره على مرحلتين وبطريقتين طريقة تم اقتراحها من قبل هذه الدراسة و الطريقة الثانية مقترحة من قبل جامعة فلوريدا. تم حقن الحيوانات المختبرية(أرناب) بالمستضد الجسمي بأربعة جرع.. تم بعد الحقنة الرابعة للحيوانات سحب الدم من الحيوان بطريقة ثقب القلب تم عزل المصل و اختبار كفاءته في الكشف عن البكتريا من خلال فحص التلازن agglutination test لمستضد البكتريا مع الضد المتمثل بالمصل و كانت النتيجة موجبة كذلك تم فحص الكفاءة النوعية للمصل من خلال اجراء فحص التلازن مع انواع بكتيرية أخرى حيث لم يعطي نتيجة ايجابية مع اي من بكتريا الاختبار. قورنت نتيجة الطريقتين أعلاه مع المصل التشخيصي المستورد وتبين ان نتيجة طريقة البحث المقترحة كانت الافضل حيث أعطت 2650 وحده/مل والطريقة الثانية اعطت 1280 وحده/مل بينما أعطى المصل المستورد 80 وحده/مل.

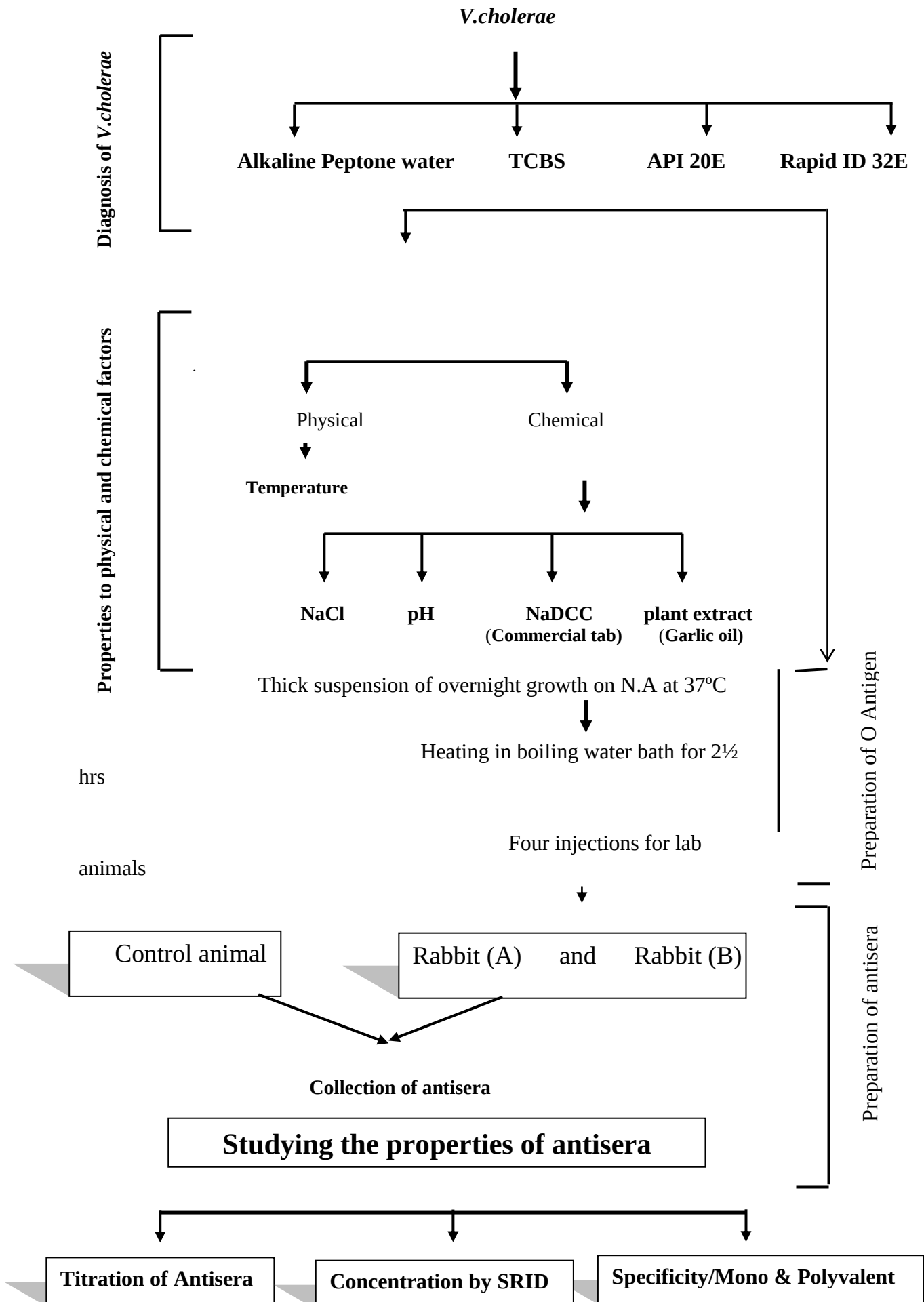
كما ان مستويات IgM ازدادت بشكل ملحوظ في الحيوانات المختبرية(أرناب A و أرناب B) حيث أعطت 3000مغ/لتر و 2400مغ/لتر على التوالي مقارنة بحيوان السيطرة 895مغ/لتر. كذلك أظهرت النتائج ارتفاعا في مستوى IgG للحيوانات المختبرية(أرناب A و أرناب B) حيث أعطت 36700مغ/لتر و 37000مغ/لتر على التوالي مقارنة بحيوان السيطرة 24500مغ/لتر.

Conclusions

- 1- *V.cholerae* can grow in alkaline media and wide range of temperature and can grow in medium with or without sodium chloride (NaCl), and no effect of garlic oil on the growth of *V.cholerae*.
- 2- *V.cholerae* can produce virulence factor represented by Haemolysin type (β) and extracellular protease enzymes other than cholera toxin.
- 3- The concentration 5.0 mg/L of sodium dichloroisocyanurate (NaDCC) for one hour is quite safe to disinfectant the drinking water.
- 4- The study confirms that Tetracycline is the most effective antibiotic against the local isolate of *V.cholerae in vitro*.
- 5- The diagnostic antisera of *V.cholerae* can be easily prepared locally. It is highly specific in diagnosis of *V.cholerae*.
- 6- The titer of locally prepared antisera from local isolate is higher and has more diagnostic value than foreign trade antisera.

Recommendations

1. The study may recommend Tetracycline as the first choice of antibiotic in the treatment of cholera in general.
2. Proceeding further studies to detect the ability of *V.cholerae* to the survival in foodstuff and water.
3. Preparation of Monovalent diagnostic antisera to differentiate between Inaba and Ogawa serotypes.
4. The study strongly recommends, depending the local antisera for diagnosis *V.cholerae* instead of other foreign trade antisera.



Schematic diagram (2-1) illustrates the main steps of the work