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Identification and Purification of Cholera Like Toxin from Environmental Isolate of *Vibrio cholerae*

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Abstract

The presence and prevalence of *V. cholerae* were investigated in forty five water samples collected from different locations of Tiger River/ Baghdad city. Twenty one isolates were isolated by adopting a simple isolation techniques. The final identification revealed that only three isolates were confirmed as *V. cholerae*. They were named 1J, 1R and Dial 131 which are all serogrouped as non-O1.

Toxin Coregulated Pili (TCP) and heat labile enterotoxin (LT) were determined in only the environmental isolate 1J while non of the isolates produced heat stabile toxin (ST).

The purification scheme was improved, few steps were adopted to include back extraction of ammonium sulfate, saturation between 80-20%, desalting through Sephadex G25, and gel filtration using Sephadex G100 which highly increase the specific activity.

Key words: environmental *Vibrio cholerae*; cholera like toxin; radial immune diffusion

Introduction

Vibrio cholerae is known to be an autochthonous inhabitant of brackish waters and esteuraine systems; there members was classified into two main serogroups, serogroup O1 their members of which agglutinate in these antisera and caused epidemics. The other serogroups of *Vibrio cholerae* , collectively referred to as non-O1 non O139, have not been associated with epidemics but can cause sporadic diarrhea and could isolated from the aquatic environment[1].

Although transmission occurs primarily through water contaminated with human feces, it is sometimes blamed for being a plausible source rather than because it has been proved responsible [2]. Epidemic cholera has been well documented in many countries as being spread by contaminated water whereas sporadic cases of cholera have been associated with the aquatic environment [3]. Contaminated water has undoubtedly been the major vehicle of *V. cholerae* transmission, with food playing a lesser but significant role in the spread of the disease Even the marine environment has been contaminated, thus making fish and other seafood gathered from the estuarines potential vehicles of transmission. Epidemic cholera caused by O1 and O139 are both produce and secrete cholera toxin (CT) which consider as the main virulence factors in cholera disease. It is fortunate that the majority of the O1 environmental strains are non-toxigenic, like most of non- O1 strains that reside in the environment, however, other toxins such as cholera toxin like toxin [4], cytotoxin, HA protease resembling enterotoxin [5], Shiga like toxin, Heat stable enterotoxin(ST), New cholera toxin

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(NCT),Sodium channel inhibitor, Thermostable direct haemolysin(TDH) Secret CHO cell elongation protein(S-CEP)may be produced by such isolates and causing sporadic diarrhea .

Many methods were adopted for the screening of the presence of CT depending either on the enzymatic activity of CT or its bioactivity saw in animal model or immunological techniques. Beside the detection of phenotypic characters depending on the production of CT, enterotoxin and ST, there are also nucleic acid methods which characterized by specificity, sensitivity and speed [6,7].

This study came to screen the production of cholera like enterotoxin , Toxin coregulated pili (TCP) and heat stable enterotoxin (ST) from local environmental isolates of *V. cholerae*; purification of cholera like enterotoxin.

Materials and Methods

Thiosulphate Citrate Bile Salt (TCBS) from Amersham and tryptone Soy agar (TSA) from Mast Diagnosis media were prepared as recommended by the manufactured while production broth (peptone 1.5, yeast extract 0.5 and NaCl 0.4, 0.2% glucose and 0.2% asparagines pH 8.5) and Lauria Birtani broth (LB) were prepared and sterilized by autoclaving at 1.5 bar for 15 min [8,9]. Diagnostic kit of Polyvalent antisera from Biomerix / France.

Samples collection and the Isolation of *V. cholerae* Forty five water samples were collected from different locations in Tiger River/ Baghdad city. They were taken from Al- Jadiriah, Al-Masbah,Al-Sarafiah Bridge, Diala Bridge, Al-Kadimia, Al-Adahmia, Al-Sinak Bridge and Salman Back. Each water samples were passed through 0.22 µm milipore filter paper then the paper was transferred to the top of TCBS medium and was incubated overnight at 37°C. Finally the yellow colonies were picked and streaked on TSA [10].

The characterization was confirmed in this study for the environmental isolates by performing morphological characterization, Microscopic examination biochemical test, and serotyping to characterize *V. cholerae* following Elliot *et al*. [11]; and WHO [12].

Detection of Toxin Coregulated Pilli (TCP):

Macroscopic examination for autoagglutination phenomenon in which visible clumping of bacteria were sew at the bottom of tube. Briefly each isolates were inoculated in 20 ml of producing broths and incubated at 35°C with aeration for 18 hour. Overnight cultures were watched for clumping and clotting of the bacterial growth [9, 13].

Extraction and concentration of enterotoxins:

Extraction and concentration of enterotoxins were achieved as described by Al- Khafaji [14].

Qualitative and quantitative detection of enterotoxins:

Qualitative detection of cholera like toxin and ST was used in which each free bacterial extract was divided into two aliquots, one of them was heat-treated at 100°C for 10 min for ST detection. For qualitative measurement two animal of Balb /c mice were injected intraperitonially (I.p) with 0.3 ml of toxin extracts. Mice were watched for 24 hour for the appearance of symptoms or death [15].

Quantitative measurement of LT was determined by measuring the erythemal activity (EA) and toxin unit was defined as each 5-8 mm of EA is equivalent to 1 Toxin Unit (TU) of enterotoxin.

TU/ml= (EA mm ÷ 5) ×10

Determination of protein concentration:

Protein concentration was measured by dye binding Bradford method in which protein concentration of unknown samples was calculated with the aid of standard curve when absorbance's of mixtures were read at 595 nm [16].

Purification of CT

purification of cholera like toxin was achieved after extraction by centrifugation for 15 min at 7000 rpm, back extraction of Ammonium Sulfate between 80% - 20% of salt saturation, desalting through sephadex G-25 and gel filtration through Sephadex G-100 to obtain purify protein following Stellwagen [17] and Al-Khafaji [9].

Radial Immune Diffusion test:

Ten milliliter of agarose was mixed with 300 µl of anti cholera toxin antibody, mixtures were mixed well without bubble formation and poured in a petridish. Only 50 µl of purified cholera like toxin was put in a hole with 3 mm diameter which made in a solidified agarose; plate was incubated at refrigerator with observation for a week for the appearance of precipitate ring [18].

Results and discussions

Vibrio cholerae infects the human intestinal tract and cause cholera disease; large numbers of *V. cholerae* are discharged in the feces of individuals. Inadequate sewage treatment allows water systems to become contaminated; in addition, there is strong evidence for long – term persistence of *V. cholerae* in environmental reservoir not directly contaminated by human feces. Consumption of water or food from such reservoirs may put a person at the risk of acquiring cholera.

Twelve samples from different locations in Dijlah River, gave turbidity cultures on APW and each culture caused sucrose fermentation changing TCBS medium to yellow. Twenty one bacterial isolates suspected *V. cholerae* were picked up from TCBS (Table1). TCBS agar is a selective medium used extensively in *V. cholerae* isolation. It suppresses bacterial species growth other than *V. cholerae,* due to alkaline pH of medium that kills most intestinal commensals and its selective ingredient suppress the growth of most interfering organisms such as Coliform, Pseudomonads, Aeromonads, and gram positive bacteria. *V. cholerae* could ferment the sucrose on TCBS medium producing yellowish colour which is nessessory to differentiate *V. cholerae* from other Vibrios [10,19]. The detection of *V. cholerae* from different samples requires three successive phases: the enrichment in a selective medium, plating onto isolating agar and presumptive identification and finally the confirmation with biochemical, serological and toxigenicity test as demonstrated by [19].

Table (1) Water samples and its location taken from Tigers River

	Sample places	Locations	Isolates
1	Al- Sarafiah bridge	Location1 Location2	/ /
2	Al-Jadiriah	Alaaras loc1 Alnahrain Univ. Alaaras loc2 Baghdad Univ. Al-Karadah	Aaras101, Aaras102,Aaras103 / / 1J /
3	Diala Bridge	Al-Rustomia loc1 Al-rustumia loc2 River side Middle of river	Rust 32,Rust 33, Rust 34 Rust 31,Rust 35 Dial 131 Dial 141,Dial 142
4	Al-Masbah	Al-Zuit factory	Mas 51,Mas 52, Mas 53
5	Al-Adalmia	Near brige loc1 Loc2	/ /
6	Al- Khadimyah	Al-Mwheet street	/
7	Salman Pack	Location1 Location2 Loc3	1R 1S,2S 4S
8	Al-Sinak bridge	Near bridge Loc2	Si91 Si92

Primary characterization was done using oxidase, string test, cholera red reaction, fermentation pattern, the production of sulpher and gas on kligler iron agar which described in table(2).

Table (2) Biochemical reactions for environmental isolates

Strain	Biochemical reactions			
	Oxidase	String test	Cholera red reaction	Kligler iron agar (slant/but)
Rust 31	+	+	+	K/A/ No gas and H2S
Rust 32	+	+	+	K/A/ No gas and H2S
Rust 33	+	+	+	K/A/ No gas and H2S
Rust 34	+	+	+	K/A/ No gas and H2S
Rust 35	+	+	+	K/A/ No gas and H2S
Mas 51	+	+	+	K/A/ No gas and H2S
Mas 52	+	+	+	K/A/ No gas and H2S
Mas 53	+	+	+	K/A/ No gas and H2S
Si 91	+	+	-	K/A/ No gas and H2S
Si 92	+	+	-	K/A/ No gas and H2S
Aaras 101	+	+	-	K/A/ No gas and H2S
Aaras 102	+	+	-	K/A/ No gas and H2S
Aaras 103	+	+	+	K/A/ No gas and H2S
Dial 131	+	+	+	K/A/ No gas and H2S
1R	+	+	+	K/A/ No gas and H2S
Dial 141	+	+	+	K/A/ No gas and H2S
Dial 142	+	+	+	K/A/ No gas and H2S
1J	+	+	+	K/A/ No gas and H2S
1S	+	+	-	K/A/ No gas and H2S
2S	+	+	-	K/A/ No gas and H2S
4S	+	+	-	K/A/ No gas and H2S

K= alkaline
A= acidic

All isolates gave positive reaction with oxidase test since all *Vibrio* species have cytochrom C as a terminal receptor for O_2 and positive to string test which confirmed by making a string like of DNA when a large loopfull of growth was suspended in a drop of 0.5% aqueous solution deoxycholate with some variation of string length. The two tests are considered as the most important diagnostic tests during the primary identification of *V. cholerae* (19).

Final identification showed that only three out of suspected isolates designed as Dial 131, 1J, 1R and represented 14% of bacterial isolates belong to non-O1 *V. cholerae* (Table 3).

Table (3) The biochemical tests for the identification of the environmental *V. cholerae*

Characters test	Environmental isolates		
	Dial 131	1 R	1J
Growth at pH 5.5	-	-	-
Growth at:			
0 NaCl	+	+	+
3% NaCl	+	+	+
6% NaCl	+	+	+
10%NaCl	-	-	-
Motility	+	+	+
Production of:			
Amylase	+	+	+
Gelatinase	+	+	+
Hemolycin	+	+	+
Lipase	+	+	+
Protease	+	+	+
MR	+	+	+
VP	+	+	+
Sensitivity to:			
lymexin B 50 IU	R	R	R
ymexin B 100 IU	R	R	R

+= positive
-= negative
R= resistant

Vibrio cholerae is known to be an autochthonous inhabitant of brackish waters and estuarine systems so aquatic environment demonstrated as the principal reservoir of *V. cholerae* where strains belong to O1and O139, non -O1 isolated from water at many part of world [20,21]. However, non-O1 strains are considered as part of the normal free living bacterial flora in Estuarines area and have been isolated from surface water at numerous sites in North America and the rates of non-O1 isolation from the environment generally exceeded those for *V. cholerae* O1 by several orders of magnitude [22].

Only *V. cholerae* 1J among the three isolates produced TCP in which bacterial culture agglutinated and sedemintated in the bottom of the culture tube and leaved clearer supernatant as presented in figure (1).

Figure (1) Autoagglutination phenomenon for *V. cholerae* 1J

cholera pathogenesis relies on a variety of carefully orchestrated factors for virulence with which TCP is used for

the colonization of the intestine mucosa and prevents vibrios washing from intestine. Also, it serves as a receptor for the phage ctxØ which harbor genes responsible for CT production and other virulence factors. Much of the investigation showed that TCP was the best characteristic pilus of *V. cholerae* and the most important thing it has presumed is that TCP like cholera toxin are exclusively associated with clinical strains of *V. cholerae*, notably those which belong to serogroup O1 and O139 (1). However, TCP has been reported among toxigenic and nontoxigenic environmental strains of *V. cholerae* [23, 24].

This investigate proved that only crude toxin extract from the environmental *V. cholerae* 1J isolate caused mice death after 24-48 hour with the appearance of some symptoms like mild diarrhea, muscle disorder, and tachycardia. I/d injection of crude extract of toxin at the shaving back of Guinea pig increased vascular permeability which caused erythema, swelling, delayed and prolonged increase in permeability of the small blood vessel of the skin leading to bleeding in injected area and induration which reached to 10mm (20 TU/ml) (Figure 2).

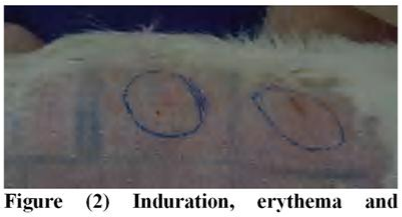

Figure (2) Induration, erythema and inflamed in skin of guinea pig due to I/d injection with cell free extract of *V. cholerae*1J.

Many authors reported that some non-O1 strains can produce CT or cholera-like toxin which may contributed to the diarrhea and other symptoms seen with wild type *V. cholerae* strains and may be responsible for the symptoms seen with non- toxigenic *V. cholerae* including the cholera- like disease or gastrointeretitis caused by toxin producer strains of non- O1 *V. cholerae* and dermatonecrotic factor which possesses pronounced cytotoxic and general toxic action, cause death of laboratory animals when entering their blood and peritonium [4,5,25,26].

No ST could be detected from the environmental *V. cholerae* 1J and that came in accordance with many investigators who mentioned that some non- O1 strains produce heat stabile enterotoxin which seemed to be responsible for diarrhea in healthy adults [27]. It was found out that only 1J *V. cholerae* from environmental isolate produced TCP and one of heat labile toxin which may be related to CT.

Precipitation and primary purification of cholera like toxin from *V. cholerae* 1J was achieved by back extraction of 80% - 20% salt saturation of ammonium sulfate which depends primarily on the assumption that inorganic salts dissolved in a large amount of water bounded to each salt molecule would lead to protein precipitate at some point when no sufficient water molecule is present to maintain solution of a set of proteins and they precipitate or salt out.

Four beaks were resolved by gel filtration chromatography using Sephade G-100 (Figure 3) in which the second beak had the biological activity causing Balb/c death and induration on Guinea pig skin.

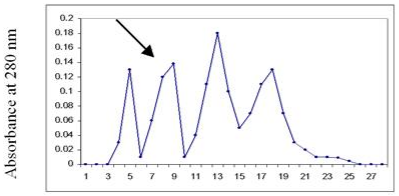

Figure (3) Cholera like toxin purification by gel filtration chromatography
SephadexG-100 column (3cm × 72cm)
Sample eluted with phosphate buffer 0.1M pH7.2
Flow rate 0.5 ml/ min.

Specific activity and purification feild of cholera like toxin increased through the purification steps. Salt - fractionation by back extraction method led to increasing specific activity and purification field reached to 17.6 which is due to the exclude of the contaminants from the crude extract. Specific activity of Cholera like toxin highly increased with gel filtration step on Sephadex G-100 to reach 710.5. The estimation of purification field revealed an increment which reached to 355 fold but the yield of Cholera like toxin decreased to only 15% and this is possibly because some of toxin protein lost its activity or suffered from subunit dissociation through purification steps as presented in table(4).

Table (4) The purification steps of Cholera like toxin from the environmental isolate *V. cholerae* 1J

Purification step	Volume (ml)	Protein concentration mg/ ml	Total protein (mg)	EA (mm)	TU/ml	Specific activity (unit/mg)	Purification field	Yield %
crude extract	125	0.04	5	5	10	2	1	100
20%-80% saturation amm.sulf.	5	0.85	4.25	15*	150	35.2	17.6	15
Desalting Sephadex G-25	12	0.2	2.2	10*	100	45.45	22.72	10
Gel filtration Sephadex G100	9	0.023	0.211	15*	150	710.5	355	15

***Sample diluted 1:5; EA= erythemal activity; TU=toxin** unit

Many precipitating rings were resolved around the purified toxin of the environmental *V. cholerae* 1J isolate (Figure 4), indicating that this environmental isolate produces similar toxin but not identical with CT . This result is in agreement with the previous toxicity result in that *V. cholerae*1J, produce one of the heat labile toxin.

Figure (4) The Radial Immune Diffusion test of purified heat labile enterotoxin from environmental *V. cholerae*1J

The presence of a common antigenic determinant between CT and heat labile enterotoxin LT produced by enterotoxigenic *E. coli* and with other enterotoxin produced by *Salmonella typhimurium* and other enterotoxin produced by *Vibrio* species other than CT were reported by others such as Dalas *et al*.(28) and Guth *et al*.(29). Also, in agreement with the work of Singh *et al*.(21) in that non-O1, non-O139 *V. cholerae* serogroups produced an enterotoxin that was antigenetically identifiable as a new toxin and this secretogenic toxin is the factor most likely responsible for enterotoxic activity saw in such strains.

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تشخيص وتنقية ذيفان شبيه بذيفان الكوليرا من عزلة بيئية لبكتريا *Vibrio cholerae*

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الخلاصة:

تم التحري عن وجود *V.cholerae* في خمس واربعين نموذج ماء لمختلف مناطق نهر دجله/بغداد وامكن عزل احدى وعشرين عزله باستخدام تقنية بسيطة للعزل واوضح التشخيص النهائي ان ثلاث عزلات فقط تعود للنوع *V. cholerae* النوع المصلي1non-O وسميت 131 1J,1RDial .

اظهر التحري عن عوامل الضراوة انتاج ذيفان عامل الالتصاق و الذيفان الحساس للحرارة من العزلة البيئية1J بينما لوحظ عدم انتاج الذيفان المقاوم للحرارة من قبل العزلات الثلاث.

طورت عملية التنقية ليشمل المخطط خطوات قليلة فيها الاستخلاص المعاكس بملح كبريتات الامونيوم بتراكيز 80-20 % وعملية ازالة الاملاح من خلال استخدام Sephadex G25 والغربلة بالهلام باستعمال هلام Sephadex G100 وقد ازدادت الفعالية النوعية لاخر خطوة بصورة كبيرة.