View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by International Institute for Science, Technology and Education (IISTE): E-Journals

Journal of Environment and Earth Science
ISSN 2224-3216 (Paper) ISSN 2225-0948 (Online)
Vol.6, No.11, 2016
www.iiste.org

Survey of occurrence of bacteria *Vibrio cholera* in the two provinces Baghdad and Babel

Kawther A. Al-Kazzaz Kadhum H. Al-Arajy

Department of Biology , College of Science , University of Al-Mustansiriya

The research is financed by Asian Development Bank.No.2006-A171(sponsoring information)

Abstract

The present study was branched into two lines ,the first line aim to study Tigris River within Baghdad city and Euphrates River within Babel city to assess physical ,chemical and biological factors of rivers water that Affect the quality of water . the second line is isolating and diagnosis of *Vibrio cholera* bacteria from clinical and environmental sources in the two provinces and study of its sensitivity against (13) antibiotic also conducting a molecular study to detect the presence of plasmids and the toxin genes in All isolated bacteria . the study area included three stations on Tigris river in Baghdad city and three stations on Euphrates river in Babel city , monthly samples were collected from October 2015 to September 2016 in addition to samples were collected from patients in three Hospitals by using sterile containers .

Keywords : Choleragen , Primers , DNA agarose gel electrophoresis

1. Introduction

Vibrio cholera is a serious pathogen for human ,has been the topic of intense researches for more than a century, is consider the most epidemiological species in the world and still endemic in some Asian countries including Iraq , this bacterium is a natural inhabitant of environment and contaminated for water and food since 1855 when John snow hypothesized that cholera disease epidemic are caused by pollution of drinking water but his theory had not been accepted , it is now recognized that cholera bacteria is naturally living in the aquatic environment and the dynamics of epidemic are related to climate and the changes in the aquatic ecosystem with seasonal cycle (Perlin et al.,2002)

The bacterium is an infection of the small intestines and the symptoms ranging from none , to mild , to severe but the classic symptom is large amount of watery diarrhea that continue from two hours to five days and causes the dehydration and imbalance in electrolytes , vomiting and cramps may also be happen . (Samuel , 1996) Unicef Organization in 2013 mentioned that new generation which is more virulent and drug-resistant strains of *Vibrio cholera* continue to appear in many areas of the world and the frequency of large epidemics with high ratios of fatality cases has increased.

Several virulence factors contribute to the pathogenicity of cholera bacteria include flagella allows the bacteria to swim across the lumen of the small intestine , Toxin co-regulated pilus (TCP) helps bacteria to adherence to the epithelial cells lining in the intestine and the TCP expression linked with the production of cholera toxin which is A-B toxin Secreted by bacteria that make the host cells discharge large amount of water and ions (Morgan et al., 2015)

The researchers studied the genetic mechanisms of the cholera bacteria to control the production of poison where the researchers discovered that there is a complex series of regulatory proteins that control on the gene expression of cholera toxin , which is called CTX that helps the bacteria to respond to the chemical environment changing in the stomach and the mucous layer of bowel wall and thus causes diarrhea (Davis et al., 2000) . PCR technique is a evolutionary method used in molecular research and based on amplifying a piece of DNA and producing of thousands to millions copies from a specific DNA sequence by used DNA polymerase (Taq Polymerase) which add a nucleotides toward 3'-OH and it require primer to add the first nucleotide to built new strand of DNA complementary to template strand , this method provided high sensitivity and specificity in Microbial identification and is helpful in the diagnosis and monitoring of epidemiological disease.(Bartlett et al.,2003)

Cholera Toxin

It is also called (Choleragen or CTX) who responsible for the deadly cholera relics and It is a model for enterotoxin , whose attaches on the walls of mucosa of the small intestine and makes it secrete a large amount of water leading to watery diarrhea characteristic of the disease (Oneal et al.,2005) in general, evolution of many pathogenic bacteria caused by horizontal transfer of genes which encode for virulence factors between organisms , cholera toxin is encoded within the genome of a filmentous phage named CTXφ (Davis et al.,2000).

Vibrio cholera has the chromosomal attachment site for integrate single stranded genome of CTXΦ , CTXΦ genome consists of a core region and repeated sequence region (RS2) ,the core region encodes for the cholera toxin A and B subunits (ctxAB) genes the main cause for the severe diarrhea that associated with cholera illness and the psh ,cep, orFU ,ace ,and zot genes which are encodes for proteins required for phage morphogenesis . RS2 region encodes for the rstR ,rstA and rstB genes needed for repression ,replication and integration of CTXΦ (Waldor et al.,1999)

In 1884 Robert Koch suggested that the symptoms caused by *Vibrio cholera* may be due to a poison it was not until 1959 when Sambhu Nath De (S.N.De) Indian scientist reported that cell-free culture filtrates from vibrio cholera (of classical biotype) when implanted directly into loops of the small intestine of rabbits could stimulate intestinal fluid accumulation (Bharati et al.,2011) In1969 Finkelstein and Lospalluto had purified the toxin and it appeared to be 84 KDa protein (Hol et al.,1995) after that other studies indicated that CT consists of two types of subunits : B subunite weighing (56 KDa) composed of five copies act as receptor binding and A subunite (28 KDa) has two segments A1 , A2 for toxic-active (Sanchez et al.,2011).

(5B) subunits binding to the specific receptors which are monosialosyl ganglioside (GM1) located on surface of the intestinal mucosa cells and a bridge piece (A2) that connect (A1) an active toxin to the (5B) subunits (Todar , 2008) when the A1 is enter into the cells of intestine it will stimulate the adenylate cyclase enzyme leading to increased levels of intracellular cAMP that activates mucosa cells to pump large amounts of H2 O , Na^+ , K^+,CL^-, HCO3 and other electrolytes from blood and tissues into lumen of small intestine which causes watery diarrhea mixed with flakes of mucus and epithelial cells and contains huge number of vibrios (Ganguly et al.,1996).

2. Materials and Methods

Materials :

A - DNA extraction kit :

Wizard Genomic DNA Purification Kit was used according to the Manufacturer's company of (Promega) for isolation of DNA .

B – Primers (Alfa DNA) :

Table (1) : The following primers were used in this study .

NO	Primer	Oligo sequence (3'-5')	Size (bp)
1	*ctx*	F5-CGGGCAGATTCTAGACCTCCTG-3 R5-CGATGATCTTGGAGCATTCCAC-3	564
2	*Zot*	F5- GGTGGCTTTTGACATGCATC -3 R5- CCAAATTGTCTACGAGGCGA -3	575

Methods :

Isolation of Vibrios from water samples :

Collect 500 ml of water sample (Treated or Raw) and filtered by using filtration unit with filter paper (pore size 0.45 µm) , and by sterilized forceps , transfer the filter paper from filtration unit and place it in tube

contain 10 ml of Alkaline peptone water media (APW) and incubated at 37℃ for 6 hours ,after that taken 0.1 ml by loop and streaking on (T.C.B.S) agar plate ,MacConkey agar and Blood agar then incubated at 37 ℃ for 24 hours . (Islam et al.,1994)

The patients samples :

Total of 11 clinical samples were isolated from patients with cholera diarrhea from three hospitals at (Fatima AL-Zahra hospital , AL-Yarmouk hospital ,Medical city hospital) for the period from 1/9/2015 to 30/10/2015 from (Male and Female), (Children and Adults).

Diagnosis of *Vibrio cholera* : by

Microscopic diagnosis , Cultural diagnosis , Biochemical tests , Api 20 E kit , Serotyping of *Vibrio cholera* and Biotyping of *Vibrio cholera*

DNA agarose gel electrophoresis :

Standard method of the (Sambrook et al.,2001) was followed to prepare agarose gel electrophoresis for Chromosomal DNA ,PCR product and Plasmid isolate : Agarose was prepared at concentration of 1% by soluble 1gram from Agarose powder in 100ml of TBE buffer and solubilized by heating with stirring then left to cool at (45-50℃) before adding (3µl) Red Safe Nucleic acid stain and poured into the gel tank with put the comb in its place to make wells and left to cool for 30 minutes .The comb was removed carefully and tank was placed in the electrophoresis system containing TBE buffer ,the buffer covers the gel for about 1-2 mm . Loaded (10 µl)of PCR product along with the DNA ladder into the wells the system of electrophoresis was covered and turned on by used 7V/cm for (1-2)hours ,the gel was examined by using UV (U.V. Transilliuminator) and then photographed .

Polymerase Chain Reaction Technique (PCR) for genes amplification :

The PCR technique was employed for amplifying Two target genes in Cholera isolates ,the primers were prepared depending on the information of manufacture company , by adding deionizer distal water (DDH2O) to get a primer concentration stock solution equal to 100 pmol/µM . Then 10 pmol/µl of working solution was prepared by taking 10 µl from the previous stock solution and completed the volume to 100 µl by adding (DDH2O) . PCR mixture and reaction conditions are summarized in table (2) and table (3) .

Table (2) : PCR mixture for target genes of *Vibrio cholera* .

Target gene	Go Taq Green MasterMix (2x)	Nuclease free water	Primers Volume (10Pmol/µl) F+R	DNA template
ctx	12.5 µl	4.5 µl	1.5 µl (from each)	5µl
zot	12.5 µl	4.5µl	1.5 µl (from each)	5µl

Table (3) : PCR reaction condition for target genes of *Vibrio cholera* .

Targe gene	Initial denaturation	No of cycles	Denaturation	Primer annealing	Elongation
ctx	94℃ /5 min	30	94℃ /30 sec	70 ℃/30 sec	72℃ /1 min
zot	94℃/5 min	30	94℃ /30 sec	60℃/1 min	72℃ /1 min

3. Results and Discussion

Sample collection (Isolation and Identification of *Vibrio cholera*) :

Cholera bacteria were isolated from two sources (water and clinical) samples , were collected (167) water samples from the Two Provinces of Baghdad and Babel as follows : Raw water (105)samples which have (23)isolate of *Vibrio Cholera* in the rate of (21 %) , Treated water (40) samples is free from Cholera bacteria , sewage water (22) samples have(8)of *Vibrio Cholera* (36%) , thus , the number of positive water isolates are (31) from the total samples (167) in the rate of (18 %) , clinical samples were collected (11) isolates from 75 stool sample from hospitals in the province of Baghdad with the rate of (14 %) , the total isolates is (42) . Other vibrios species were isolated from water samples and identified by cultural , biochemical tests and API 20E system as : *V . fluvialis* (26%) ,*V .parahaemolyticus* (14%) , *Aeromonas hydrophila* (15%) .

Detectin of Cholera toxin by PCR technique :

Genomic DNA was extracted from All *Vibrio cholera* isolates and the range of DNA concentration was 50-100 np /ml and the DNA purity was 1.7 – 2.1 ,this quantity and purity of DNA extraction are enough for amplification by PCR In this study was determined the presence of virulence genes which encode for cholera toxin among the clinical and environmental isolates of *Vibrio cholera* and PCR technique was performed to detect the existence of toxin genes is *ctx* and *zot* genes. The results showed that all of the clinical isolates were owned a toxin genes in the rate (100%) while the environmental isolates were free from it . The results agreed with the local study of Al-Thwani in 2007 that all clinical isolates of O1 with El tor biotype was contain the virulence gene *ctx* while the environmental isolates of Non O1 serotype was free from gene , and the results agreed with Bakhshi et al in 2008 when PCR assay has been used to confirm the presence of virulence genes *ctx* ,*zot* and *ace* in clinical and environmental isolates of vibrio cholera in Iran and the conclusion is absence of all virulence genes from the environmental isolates while the clinical isolates were acquired all set of genes which turn them into pathogenic strains Wehmeyer et al.,2016 explained When the bacteria enter human body, will stopped production of some proteins and activate the production of other regulatory proteins that helps in adaption to the chemical environment of the stomach until it reaches the intestines and inhabit in it , one of the regulatory proteins is ToxT which control the expression of cholera toxin genes that causes watery diarrhea .

L 1 2 3 4 5 6 7 8 9 **L** 10 11 12 13 14 15 16 17 18

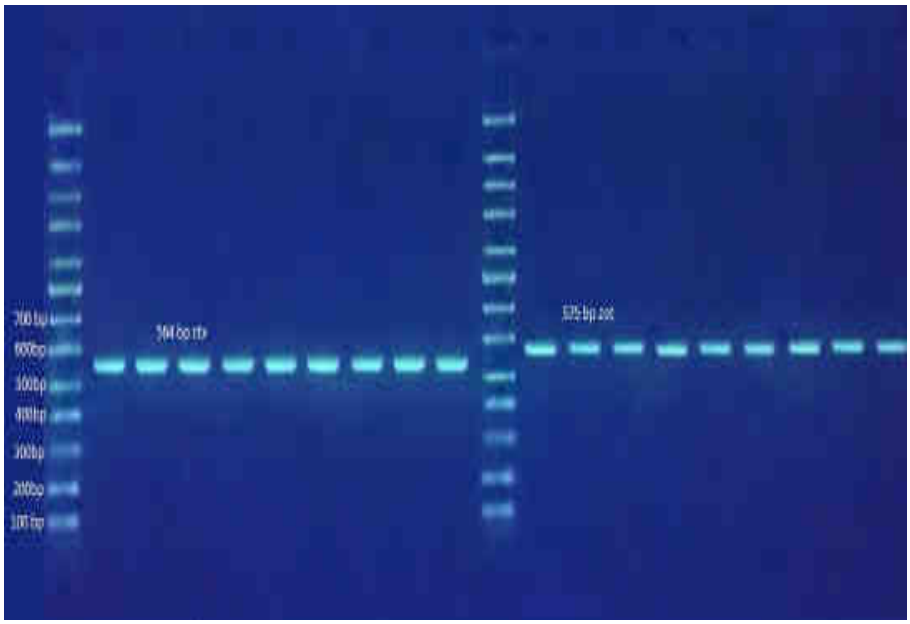

Figure (1) : Agarose gel electrophoresis (7 V/cm , 2 hours) for PCR products show Clinical isolates of *Vibrio cholera* (Inaba) , (1 - 9) lines contain toxin gene (ctx : 564 bp) , (10-18) lines contain toxin gene (zot : 575 bp) , L line is DNA ladder 100 bp .

Table (4) : Biochemical tests for Vibrios species

Biochemical test	**V . cholera**	**v. fluvialis**	**V. parahaemolyticus**	**Aeromonas hydrophilia**
TCBS agar	Yellow	Yellow	Green	Yellow
KIA	Ka	KK	KA	KK
Oxidase	+	+	+	+
Indole test	+	-	+	+
Cholera red	+	+	+	+
Voges-Proskauer	+	-	-	+
Citrate	+	+	+	+
Motility	+	+	+	+
String test	+	+	-	-
Urease	-	-	V	-
ADH (from Api test)	-	+	-	+
ODC (from Api test)	+	-	+	-
LDC (from Api test)	+	-	+	+

TCBS : Thiosulphate citrate bile salt , KIA : Kligler iron agar , ADH : Arginine dihydrolase , ODC : Ornithine decarboxylase , LDC : Lysine decarboxylase , V : Variable , KK : Slant alkaline / Butt alkaline , KA : Slant alkaline /Butt acidic , Ka : Slant alkaline / Butt slightly acidic

Reference

Al-Thwani , A.N . ; Al-Khafaji , KH. (2007) . Diagnosis of some virulence factors toxigenic in clinical and environmental isolates of Vibrio cholera . Genetic Engineering Institute . University of Baghdad .

Bartlett , J.M. ; Stirling , D. (2003) . A short history of the polymerase chain reaction PCR protocols . Methods in Molecular Biology . P : 3-6 .
Bharati , K. ; Ganguly , N.K. (2011) . Cholera toxin : A paradigm of a multifunctional protein . Vol. 133(2) . P : 179-187 .
Bakhshi , B. ; Pourshafie , M.R. (2008). Comparison of distribution of Virulence determinants in clinical and environmental isolates of Vibrio cholera . Vol. 12(3) . P : 159-165 .

Davis , B.M. ; Moyer , K.E. (2000) . CTX prophages in classical biotype Vibrio cholera : Functional phage genes but dysfunctional phage genomes . J. Bacterial . Vol 182(24) . P : 6992-6998 .
Ganguly , N.K. ; Kaur , T. (1996). Mechanism of action of cholera toxin and other toxin . Indian . J. Med . Res . Vol. (104) . P : 28-37 .
Islam , M.S. ; Drasar , B. S. ; Sack , R. B.(1994). The aquatic flora and fauna as reservoirs of Vibrio cholera : a review . Vol.12(2) . P : 87-96. J. Diarrheal . Dis . Res .
Morgan , S.J. ; French , E.L. (2015) . Formation of an intra molecular periplasmic disulfide bond in TcpP protects TcpP and TcpH from degradation in Vibrio cholera . Vol. (3) . P : 498-509 .
Oneal , C.J. ; Jobling , M.G. ; Holmes , R.K. (2005) . Structural basis for the activation of cholera toxin by human . J . of Science . Vol.309 (5737). P : 1093 – 1096 .
Perlin , D . ; Cohen , A . (2002). Water-borne disease : Cholera and Dysentery , The complete idiot's guide to dangerous disease and epidemics (USA) .
Sambrook , J.;Russel , D .(2001). Molecular Cloning : A Laboratory Manual Gold Spring Harbor laboratory . New York .

Sanchez , J. ; Holmgren , J. (2011). Cholera toxin –Afoe and a friend , Indian , J. of .Medical . Res . Vol.(133). P : 153-163 .
Samuel , B. (1996). Medical Microbiology , 4 th edition , University of Texas , Medical Branch at Galveston . Page . 8
Todar , K. (2008). Vibrio cholera and Asiatic cholera , Vol.(1) . P : 1-4
Unicef , (2013) . Cholera ToolKit , Unite for Children , New York ,USA .
Wehmeyer , G.; Lovell , S.(2016).A resolution structure of the AraC-family transcriptional activator ToxT from Vibrio cholera vol.72(9). P : 726-731 .
Waldor , MK . ; Boyd , EF. (1999) . Alternative Mechanism of cholera Toxin acquisition by Vibrio cholera : Generalized transduction of CTXΦ by Bacteriophage CP-T1 , Vol. (67) . P : 5898-5905 .
Hol , W.G.J. ; Sixma , T.K. ; Ethan , A. M. (1995) . Structure and function of E.coli Heat – Labile enterotoxin and cholera toxin B pentamer . P : 186-215 .