

## COMPARATIVE STUDY BETWEEN *VIBRIO CHOLERA* ISOLATED FROM PATIENTS AND FROM SURFACE WATER IN IRAQ USING PULS FIELD GEL ELECTROPHORESIS

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Received 25/4/2010

Accepted 11/10/2010

### ABSTRACT

The current work include the molecular epidemiological study of two types of *V. cholera*, the clinical isolates (9 isolates) which have been taken from laboratory health center-Baghdad, during 2004-2006 and the environmental isolates(5 isolates), recovered from surface water in Baghdad and some governorates by using two methods, the first one the isolation and electrophoresis of plasmid DNA through Gel electrophoresis, the second, the isolation and electrophoresis of chromosomal DNA using PFGE for the detection source of Cholera infection. Results revealed eight clinical isolates were *V. Cholera* type O1 and subtype Ogawa and Inaba, only one of them was type non O1, while all the environmental isolates were non OI type, beside, all isolates from O1 belonged to El-Tor type. The results of DNA investigation revealed the absence of DNA plasmid in clinical and environmental isolates. While the chromosomal analysis using PFGE showed normal distribution of cutting chromosomes digested by restriction enzyme not I revealing nine bands of DNA of Cholera subtype Inaba, O1 and Altor type with molecular weight ranging 50-425 kb base. No investigation done for Cholera subtype Ogawa which had been isolated from patient, because it appears like a smear on the gel used.

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Key words: *Vibrio cholera*, Surface water, Puls field Gel Electrophoresis

## دراسة مقارنة بين ضمات الكوليرا المعزولة من مرضى الكوليرا وتلك المعزولة من المياه السطحية في العراق باستخدام جهاز الترحيل الكهربائي ذا الحقل النبضي

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القبول 2010/10/11

الإستلام 2010/4/25

### الخلاصة

تضمن البحث دراسة وبائية جزيئية لبكتريا الكوليرا بنوعها العزلات السريرية (9 عزلات) والتي تم الحصول عليها من مختبر الصحة المركزي - بغداد خلال سنة 2004-2006 ، والعزلات البيئية (5 عزلات) والتي تم الحصول عليها من المياه السطحية من بغداد وبعض المحافظات و بإستعمال طريقتين : الأولى عزل وترحيل DNA البلازميدي بواسطة الترحيل الكهربائي والثانية عزل وترحيل DNA الكروموسومي بواسطة الترحيل الكهربائي ذا الحقل النبضي (PFGE) لغرض الكشف عن مصدر الإصابة بمرض الكوليرا. أظهرت النتائج إن 8 من العزلات السريرية كانت ضمات الكوليرا ذات النمط المصلي O1 ونمط تحت المصلي Ogawa و Inaba وأن عزلة واحدة فقط كانت ذات نمط مَصلي Non O1 ، بينما كانت كل العزلات البيئية ذات نمط مَصلي NonO1، كما أن جميع العزلات من O1 تعود للنمط الحيوي الطوري. وأكدت نتائج فحص DNA عدم وجود DNA بلازميدي في العزلات السريرية و البيئية، بينما أعطى التحليل الكروموسومي بواسطة التحليل الكهربائي ذا الحقل النبضي توزيعاً مناسباً للقطع الكروموسومية المهضومة بواسطة الأنزيم القاطع Not I لينتج 9 حزم من DNA للضمات ذات نمط تحت المصلي Inaba, O1 والنمط الحيوي El-tor وبحجم جزيئي يتراوح بين 425-50 كيلو قاعدة، بينما لم يتم تحليل نتائج الضمات ذات النمط تحت المصلي Ogawa والتي عزلت من المرضى لأنها ظهرت بشكل مسحة على الهلام المستخدم.

## INTRODUCTION

Cholera is an acute intestinal infection caused by the bacterium *Vibrio cholerae*, which produces an enterotoxin that causes copious, painless, watery diarrhea that can quickly lead to severe dehydration and death if treatment is not promptly given. Vomiting also occurs in most patients. When cholera occurs in an unprepared community, case-fatality rates may be as high as 50% usually because there are no facilities for treatment, or because treatment is given too late (1).

More than half of the world's populations live in cholera endemic areas; previously the disease swept the world in six great pandemics (1817-1923) and later receded into its ancestral home in the India-Pakistan subcontinent (2,3).

In 1965-1966 the El Tor biotype was transmitted from Asia and Middle East to Iraq and Iran through trading, in the seventh pandemic (4). During the past three to four years, serious outbreaks have occurred in different parts of Iraq, sometimes involving antibiotic resistance strains. In the past the ability to differentiate individual strains of *V. cholerae* for epidemiological purposes has been hampered by the low discriminatory capability of available typing systems, such as biochemical and serologic identification, in view of this, newer approaches to molecular epidemiology should be considered as potentially important tools in the effective monitoring of strains of *V. cholerae* especially those associated with epidemiologically well-defined outbreaks. With regard to the application of molecular techniques in *V. cholerae* epidemiology, pulsed field gel electrophoresis (PFGE) and also plasmid profile analysis, have been used to analyze *V. cholerae* strains associated with cholera outbreaks. Pulsed Field Gel Electrophoresis (PFGE) separates large DNA fragments created by digestion of total genomic DNA with restriction endonucleases that cut DNA infrequently (5).

Depending on our best knowledge, till now, no study have been performed in Iraq concerning the molecular and epidemiological relationship between clinical and environmental *V. cholerae*; therefore. This study aims to isolate and identify the pathogen from patients and from environment in order to clarify this relationship between them and attempted to determine the main source of the disease.

## MATERIAL AND METHODS

### Bacterial Strains

#### Clinical Isolates

The clinical *V. cholerae* isolates were gained from the Central Health Laboratories in Baghdad which were isolated in a period from Aug. 2004-Nov. 2006.

#### Environmental isolates

A total of 100 water samples were collected from river of five different provinces in Iraq Table (1), in a period from August 2004 to November 2005.

By using the membrane filter technique, a 500-1000 ml of water samples were collected, concentrated on 0.22  $\mu$ m Pore-Diameter filters; this was followed by incubation of the membranes in an enrichment medium of alkaline peptone water (APW) over night at 37 °C.

A loopful of the culture broth, was taken from the top layer of the APW then streaked on to TCBS agar and incubated overnight at 37 °C. Two-three yellow, flat, 1-3 mm diameter isolated colonies were picked from each sample and streaked on tryptic soy agar (TSA) and MacConkey agar for further testing (6).

**Table (1) : Regions of collecting water samples.**

Provinces	Sectors
Baghdad	Al-Jadyria / Abu-Graib / Al-Adamyia / Al-Shawaka/ Selman pak
Babel	Al-Haswa
Wasit	Al-Sewera
AL-Basrah	Shat al Arab
Al-Sulaymania	Quliasan / Sarchnar

The clinical and environmental isolates identified by routine biochemical tests, Api 20 E system, serological and biotypic tests (7).

### **Molecular Characterization Tests**

#### **A- Plasmid Profile by Gel Electrophoresis**

Ten of *V. cholerae* isolates were screened for plasmid content by the alkaline method of (8).

#### **B- Chromosomal profile by PFGE**

Which was conducted by many steps as following:

##### **A-Preparation of DNA plugs**

Cultures were incubated in 15 ml of HIB at 37 °C overnight until growth reached to 10<sup>9</sup> bacterial cells (optical density of 0.6 at 610 nm for *V. cholerae*) then ten ml of cultured broth were harvested by centrifugation and were washed with 10 ml of wash buffer(5,9). Cells were resuspended in 1 ml of wash buffer and warmed at 37 °C for few minutes, then mixed with an equal volume of 1% low melting agarose after that they were dispensed into a plug mold.

Agarose plugs were allowed to solidify on ice for 10 min and plugs were removed from mold and placed in clean tubes containing 300 µl of lysis buffer for each 100 µl plug. Bacteria were lysed in the agarose plugs for 1 hour at 37 °C, then the plugs were incubated overnight in 300 µl ESP buffer for each plug at 50°C, the plugs were rinsed briefly with deionized water, in the next day and washed twice in 200 µl of the TE buffer containing 30 µl of 0.1 M PMSF for 30 min each time after that they were washed four times in 300 µl of the TE buffer for 30 min each time.

A small portion of the plug (2 by 7 mm) was sliced off and incubated for 1 hour in a micro centrifuge tube in 1 µl of *Not I* buffer.

The buffer was replaced with 125 µl of fresh buffer containing 20 U of *Not I*, and the mixture was incubated for 4 hours at 37 °C.

#### **B-Separation of *Not I* restriction fragments by Gene navigator system (PFGE) (10)**

#### **C-PFGE patterns:**

The goal of the interpretative process is to use the typing data to predict the clinical and environmental relationships among isolate, Table (2) by Tenover *etal.* (11) shows how to interpretate PFGE patterns.

**Table (2) : Criteria for interpreting PFGE Pattern (11)**

Category	No. of Genetic differences compared with outbreak strain	Typical No. of fragments differences compared with outbreak strain	Epidemiologic interpretation
Indistinguishable	0	0	Isolate is part of outbreak
Closely related	1	2-3	Isolate is probably part of outbreak
Possibly related	2	4-6	Isolate is possibly part of outbreak
Different	>3	>7	Isolate is not part of outbreak

## RESULTS AND DISCUSSION

### Isolation and Identification of Clinical and Environmental Bacteria:

Nine clinical isolates of *V. cholerae* were gained from Central Public Health Laboratories (CPHL) in Baghdad, and were designed as C1, C2, C3, C4, C5, C6, C7, C8, and C9. Their identification was confirmed according to Stavric and Bachanan.1995 (7), by routine biochemical tests and Api 20 E system, in addition to cultural and microscopic tests. The serological results revealed that, eight of them were O1 and one isolate was Non/O1. These obtained were isolated from stool of patients with profuse watery diarrhea in different periods of 2004 and 2006.

Twenty nine bacteria of different genera and species were isolated and identified from water samples which included, *Aeromonas hydrophila*, *Vibrio valnificus*, *Vibrio parahaemolyticus*, *Vibrio fluvialis*, and *Vibrio hemolyticus*, in addition to *V. cholerae* Non/O1 which represented 5 % and were designed as E1, E2, E3, E4, and E5 Table(3).

Table(3): Biochemical tests of clinical and environmental *Vibrio cholerae*

Year of isolation	Iso.	Oxidase test	String test	Cholera red	KIA	TSI	T1N0	T1N6	T1N8
2004	C1	+	+	+	K/A	A/A	+	v	-
	C2	+	+	+	K/A	A/A	+	-	-
	C3	+	+	+	K/A	A/A	+	-	-
	C4	+	+	+	K/A	A/A	+	v	-
	C5	+	+	+	K/A	A/A	+	-	-
	C6	+	+	+	K/A	A/A	+	-	-
	C7	+	+	+	K/A	A/A	+	-	-
	C8	+	+	+	K/A	A/A	+	-	-
2006	C9	+	+	+	K/A	A/A	+	v	-
2004	E1	+	+	+	K/A	A/A	+	-	-
	E2	+	+	+	K/A	A/A	+	-	-
	E3	+	+	+	K/A	A/A	+	-	-
	E4	+	+	+	K/A	A/A	+	v	-
2005	E5	+	+	+	K/A	A/A	+	-	-

(C P Clinical isolates, E P Environmental isolates, += positive, - = negative, v = variable).

Its now evident that the optimum growth of *V. cholerae* is in the warm climate (12,7,13), this piece of information supports the results of the present study which revealed that the best month for isolating *V. cholerae* was in August due to its suitable warm climate for the

pathogen's growth and multiplication, similarly, (14,15) reported that the most outbreaks of cholera disease occurred in summer season, but our results disagreed with the previous observations (16) which recorded that in Iraqi epidemic of 1991, and the inter-epidemic period, the number of clinical cases typically peaked during the autumn months, especially September and October.

#### **Plasmid profile of *V. cholerae*:**

The current study demonstrated that no plasmids were found in all of clinical and environmental isolates which disagreed with the results obtained (15) who could isolate one plasmid in 40 % of isolates. On the other side (17) have reported that, there weren't any plasmids in *V. cholerae* O1 and Non/O1 that were isolated in Iran. This provided better indication that isolates of this study may be of the similar origin, moreover although our isolates contained no plasmids but they were resistant to streptomycin, cephotaxime and amikacine, and this was explained by (18) who found that the corresponding resistance genes are located on large conjugative elements that are integrated into *V. cholerae* chromosome, these genes are clustered within a composite transposon – like structure found near the elements 5' end.

#### **Chromosomal Profile by PFGE:**

PFGE is a useful tool for epidemiologic surveillance of *V. cholerae*, in human, *V. cholerae* infection results from ingestion of the organism, usually with contaminated food or water, thus, the goal of strain typing studies is to provide laboratory evidence that, epidemiologically related isolates collected during an outbreak of disease are also genetically related and thus represents the same strain and that give a good indicator for the source of infection. This information is helpful for understanding and controlling the spread of disease in both hospitals and communities (11).

PFGE provided an excellent tool for rapidly examining the genomes of various serovars and biovars of *V. cholerae* and to determine the precursor of the disease caused by those bacteria, so in this study we tried to analyze Ten isolates by PFGE, eight of them were clinical *V. cholerae* O1 (2 Inaba and 6 Ogawa) and the other two were Non/O1 *V. cholerae* (1 environmental and 1 clinical isolate) and tested only one environmental isolate which was isolated from Al-Jadyria (E5) and represented other environmental isolates to be compared with the clinical *V. cholerae* of the same serotype (Non/O1).

PFGE analysis with the restriction enzyme Not I (5'-GCGGCCGC-3') gave a suitable distribution of fragments, with about nine DNA fragments of *V. cholerae* O1 subtype Inaba, biotype El Tor, ranging in size from 50-425 kb as shown in figure (1), and about DNA fragments of *V. cholerae* Non/O1 (Nag) ranging in size from 125-425 kb as shown in figure (2). There were no any Iraqi references to compare these results with, therefore, we relayed on many researches that have been studied *V. cholerae* strains in order to analyze our findings.

(11) Suggested that, when the restriction enzyme *Not I* is used in digesting the bacterial chromosome, it will reproduce 20-30 DNA fragments and 10-400 kb.

The Non/O1 *V. cholerae* produced 8 DNA fragments and a ranging band size 50-425 kb, but the results of (19) study have showed (15-21) DNA fragments with ranging size (97-485 kbp). These differences between Sharma patterns and our pattern were for the same reason mentioned above, i.e., setting multi phase ramping time, in addition to that, they used (50 U) of Not I restriction enzyme for one hour while we used (20 U) of Not I for four hours.

The PFGE analysis of *V. cholerae* O1 subtype Ogawa, which was isolated from patients, could not be typed and consistently appeared as a smear on the gel, and the reason could be due to mistakes in preparing the plugs or mistakes in loading of the samples or it was due to the use of inappropriate parameters which may affect the resolution and the quality of its DNA bands, as field strength which has a profound effect on pulsed-field separations and is a compromise between separation time and resolution of particular size class, generally (4-6 volts/cm) is required for resolving DNA up to (2 Mb) in reasonable period of time (1-2) days(20).

DNA Quality and concentration may be one of the reasons the concentration of DNA affects its mobility and, hence, the clarity of bands and also the ease with which comparison of banding patterns can be achieved both by eye and by computer-assisted analysis (21), in any case the last reason could be a good one to explain the smear that have been reproduced.

The interpretation of results of the isolates' PFGE pattern were as following; the two Inaba isolates that were isolated from patients in the year 2004 were Indistinguishable, because they had an identical number and size of DNA fragments, Epidemiologically related, because these isolates were obtained from patients during a discrete time frame and from a well-defined area as a part of an epidemiological investigation that suggests that the isolates may be derived from a common source, An outbreak strains, because they are both epidemiologically and genetically related (i.e. have indistinguishable genotypes) so these assumed to be clonally related since they have common phenotypes and genotypes and were isolated within a defined period, but, not an endemic strains because an endemic strains should not have a direct or epidemiologic linkage, although they are indistinguishable by typing methods, in other words, the endemic strains are clonally related but their common origin may be more temporally distant from those of outbreak strains.

Both Non/O1 *V. cholerae* were isolated from Baghdad, but one of them was isolated from patient in 2006 and the other one was isolated from surface water in 2005 as lane-1 and lane-2 show in figure (2) respectively these isolates were; Indistinguishable, so they are genetically related, but, epidemiologically unrelated, because they were isolated at different times that means they are NOT an outbreak strains because they are epidemiologically unrelated, but, they are an endemic strains because they are clonally related and may be the water is the source of infection.



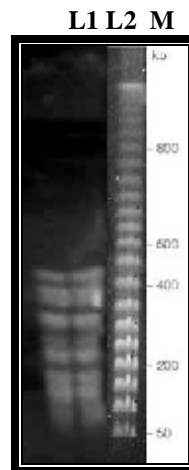


Figure (1): The PFGE pattern of *V. cholerae* subtype Inaba shows 3 lanes; L1 represents C1 isolate with 9 bands, L2 represents C2 with 9 bands, and M represents the lambda ladder marker (1000 kb).

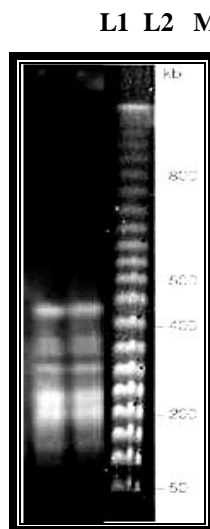


Figure (2): The PFGE pattern of Non/O1 *V. cholerae* shows 3 lanes; L1 represents C9 isolate with 8 bands L2 represents E5 with 8 bands, and M represents the lambda ladder marker (1000 kb).

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