Toxigenic Potential of *Vibrio cholerae* O1and O139 Serotypes Isolated from Cases of Diarrhea in Baghdad hospitals

سمية ضمات الكوليرا الأنواع المصلية المعزولة O1 وO139 من حالات الإسهال في مستشفيات بغداد

Ibtesam Ghadban Auda **Israa Mohamed safi al- kadmy**
College of science /Al –Mustansiriyah University
ابتسام غضبان عودة **إسراء محمد صفي الكاظمي**
كلية العلوم /الجامعة المستنصرية

Abstract

Cholera is most important water borne pathogen. The public health significance of a *V. cholerae* isolate is routinely assessed by two critical properties: the production of cholera toxin CT and the possession of either the O1 or O139 antigen, which acts as a marker of epidemic potential. The objective of this study is to detect *V. cholerae* serotypes directly from stools and determines their toxiginicity potential. Sixty four stool samples were collected from four hospitals in Baghdad from November 2010 to February 2011. The age of patients was ranging from two months to 12 years, 26 females and 38 males. Immunochromatographic test used for qualitative detection of O1and /or O139 serotypes was used in addition to routine culture for isolation of *V. cholerae*. Using specific primer cholera toxin gene, *ctxA2-B,* was amplified and the PCR product was detected by agarose gel electrophoresis. Out of 64 stool samples only 16 (25%) was positive. Fifeen 93.7% of these samples were positive for O1serogroup and just one 6.3% was positive for O139 serogroup. Stool sample culture on alkaline peptone water and then on TCBS agar enhance the growth of 11(17.2%) *V. cholerae* isolates, 10 (90.9%) were belong to O1 serotype and one 9.1% belong to O139. The results of *ctxA2-B* gene amplification show that, 9 (90%) out of 10 O1serotypes was positive. While the only one 100% O139 serotype was positive. As conclusion, the incidence of cholera caused by *V. cholerae* O1 is more than that caused by *V. cholerae* O139 in Baghdad hospitals. Immunochromatographic test is a rapid and sensitive test in recover *V. cholerae* O1 and O139 serotypes. PCR is a simple molecular tool to determine the toxigenicity of *V. cholerae* isolates.

Key words: *Vibrio cholera,* O1 or O139 antigen

المستخلص

تعتبر الكوليرا من الامراض المهمة وان اهميتها في مجال الصحة العامة تاتي من صفتين الاولى افراز ذيفان الكوليرا وامتلاك احد المستضدين O1 او O139, ان الغرض من هذه الدراسة هو ايجاد طريقة سريعة للكشف عن وجود بكتيريا الكوليرا مباشرة من البراز والكشف عن سميتها. تم جمع 64 عينة خروج من اربع مستشفيات في بغداد في الفترة من تشرين الثاني 2010 الى شباط 2011 تراوحت اعمارهم بين شهرين و 12 سنة 26 انثى و38 من الذكور, استعمل اختبار الكروموتوكرافيا المناعي المرئي لتشخيص الانماط المصلية بالاضافة الى استعمال طريقة الزرع الروتيني على الاوساط الزرعية.واستعملت تقنية التفاعل التسلسلي لانزيم البلمرة للكشف عن وجود جين ذيفان الكوليرا, 16(25%) من عينات الخروج اعطت نتيجة موجبة كانت منها 15(93.7%) تابعة للنوع المصلي O1 بينما كانت واحدة فقط 6.3% تابعة للنوع O139. اما طريقة الزرع الروتيني فانها اظهرت نمو في 11(17.2%) فقط كانت منها 10(90.9%) تابعة للنوع المصلي O1 وواحد 9.1% تابعة للنوع O139. كانت نتائج التفاعل التسلسلي لانزيم البلمرة للكشف عن ذيفان الكوليرا كما ياتي : 9(90%) من النوع المصلي O1 كانت تحوي على هذا الجين وكذلك العزلة الوحيدة التابعة للنوع O139 (100%). نستنتج من هذه الدراسة ان النوع O1 كان اكثر شيوعا من O139 وان اختبار الكروموتوكرافيا المناعي المرئي اختبار حساس للكشف عن بكتيريا الكوليرا وان اختبار التفاعل التسلسلي لانزيم البلمرة هو وسيلة جزيئية سهلة في الكشف عن قابلية البكتيريا على انتاج الذيفان .

الكلمات المفتاحية: الكوليرا، المستضدين O1 او O139

Introduction

Vibrio cholerae is a facultative anaerobic, Gram negative, non-spore forming curved rod. It is a human pathogen found in coastal waters that causes the acute gastrointestinal disease. Cholera is a major health threat in poor nations. It is widely acknowledged as one of the most important water borne pathogen[1]. The threat of epidemic cholera is restricted primarily to developing countries with warm climates [2]. There are 139 different O groups. *V. cholerae* O1 and O139 are known to be the dominating and pathogenic strains [1]. The public health significance of a *V. cholerae* isolate is routinely assessed by two critical properties: the production of cholera toxin (CT) and the possession of either the O1 or O139 antigen, which acts as a marker of epidemic potential. So far, agents of endemic and pandemic cholera

have been represented exclusively by CT-producing *V. cholerae* strains. Cholera toxin has been shown to be the key virulence factor responsible for the manifestation of massive, dehydrating diarrhea [2,3,4]. The pathogenicity of *Vibrio cholerae* is chiefly associated with the secretion of the CT, which is a protein complex.

The structure of CT is typical subunit group of toxins in which each of the subunits has a specific function [1]. The A subunit functions for adenylate cyclase activation in small intestinal epithelial cells, leading to the loss of fluid and electrolytes. The binding (B) subunits of 11500 Da each serves to bind the toxin to the epithelial cell surface receptor [5]. The catalytic A subunit must gain access to the cell cytosol for CT to exert its toxic effects [6]. The genes expressing A and B subunits are designated *ctxA* and *ctxB*, respectively, and are expressed as a single transcriptional unit [5].

Detection of CT-producing *V. cholerae* using conventional culture-, biochemical- and immunological-based assays is time-consuming and laborious, requiring more than three days. A rapid, reliable and practical assay for the detection of CT-producing *V. cholerae* has thus been sought. Several PCR assays offer a more sophisticated approach to the identification of *V. cholerae* [7].

Although PCR assays provide more rapid identification of *V. cholerae* than conventional assays, they require the use of electrophoresis to detect amplified products, which is time-consuming and tedious. Real time PCR assays recently developed for the rapid identification of *V. cholerae* [8]. Real time PCR assays are not routinely used due to their requirement for an expensive thermal cycler with a fluorescence detector [9,10]. PCR has now become a frequently used detection method, and several PCR protocols have been developed for *V. cholerae* [11,12,13].

In this study we investigate the incidence of *V. cholerae* O1and O139 serotypes for diarrheal cases in Baghdad city and their toxigenic potential.

Materials and methods

Sixty-four stool samples were collected from four hospitals, Al-Kadhymia teaching hospital, Al-Kadhymia hospital for children, Children protection hospital in Al-Mansur neighbourhood and Baghdad teaching hospital. The age of patients was ranging from two months to 12 years, 26 females and 38 males.

Immunochromatographic one step visual test for *V. cholerae* (Crystal VC-India), for qualitative detection of O1and /or O139 serogroups was used. This test based on the principle of immunochromatographic, in which the nitrocellulose membrane is coated with monoclonal antibodies to *V. cholerae* O1and O139 LPS as two separated bands.

Alkaline peptone water was prepared as an enrichment broth, as follows 10g of peptone and 10g of sodium chloride were dissolved in 800 ml of distilled water, the pH was adjusted to 8.5 then the volume was completed to 1000 ml then autoclaved. Thiosulfate citrate bile salts sucrose (TCBS) agar is the selective agar medium of choice for isolating *V. cholerae.* Ten milliliters of alkaline peptone water were inoculated with about 1 ml of stool sample that was positive when tested by Immunochromatographic one step visual test and then incubated 6 hrs at 35-37°C. After 6 hrs of incubation, about 0.1 ml was inoculated on the surface of TCBS Agar [14].

The total DNA of *V. cholerae* O1and O139 serotypes were extracted by simple and rapid boiling procedure. Briefly, portions of individual bacterial colonies were suspended in 200 µl of lysis buffer containing 1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl (pH=8.0), and 1 mM EDTA and incubated for 3 minutes in a boiling water bath. After centrifugation for 2 minutes at 10,000 ×*g* to sediment the debris, a 10-µl aliquot of the clear supernatant was directly used for agarose gel electrophoresis [15] and 5-µl aliquot transferred to the PCR Master mix of PCR [16].

The sequence of oligonucleotide primers that were used in PCR to detect the presence of *ctxA2-B* gene were taken from [17] and synthesized in Alpha DNA Co. (Canada). Table (1) showed primers sequence and their PCR product. This gene was amplified using PCR, 5-µl aliquot of the DNA supernatant was directly transferred to the PCR Master mix as a template. The amplification was performed as follows (according to the manufacturer instruction- Promega-USA):

Go-Taq green master mix	**12.5 µl**
Each primer (set of 10 picomol\µl each)	**1.5 µl**
Nuclease free distilled water	**4.5 µl**
DNA template	**5µl**

The PCR reaction was performed with a denaturing step at 94 °C for 5 minutes, followed by 30 cycles at 94 °C for 45 seconds, 52 °C for 50 seconds, and 72 °C for one minute. Sterile distilled water was used instead of DNA template to ensure absence of contaminants in the reaction preparations [17].

Table (1): The sequence of *ctxA2-B* gene specific primer set and its product size

primer	Sequence (5´ 3´) →	*gene*	PCR product
C2F	AGGTGTAAAATTCCTTGACGA	*ctxA2-B*	385bp
C2R	TCCTCAGGGTATCCTTCATC		

Results

Immunochromatographic one step visual test figure (1) shows that, out of 64 stool samples only 16 (25%) was positive. Fifeen 93.7% of these samples were positive for O1serogroup and just one 6.3% was positive for O139 serogroup. Stool sample culture on alkaline peptone water and then on TCBS agar enhance the growth of 11(17.2%) *V. cholerae* isolates, 10 (90.9%) were belong to O1 serotype and one 9.1% belong to O139 Table (2,3).

Table (2): Number and percentage of positive and negative *V. cholerae* O1 and O139 serotypes using two methods.

No of stool samples	Immunochromatographic test			Culture		
	Negative	Positive		Negative	Positive	
		O1	O139		O1	O139
64	48	15	1	53	10	1
	(75%)	(25%)		(82.8%)	(17.2%)	

Table (3): Number and percentage of positive *V. cholerae* O1 and O139 serotypes using two methods.

V. cholerae serotypes	Positive stool culture	Percentage (%)	Positive Immunochromatographic test	Percentage (%)
O1	10	90.9	15	93.7
O139	1	9.1	1	6.3
Total	11	100	16	100

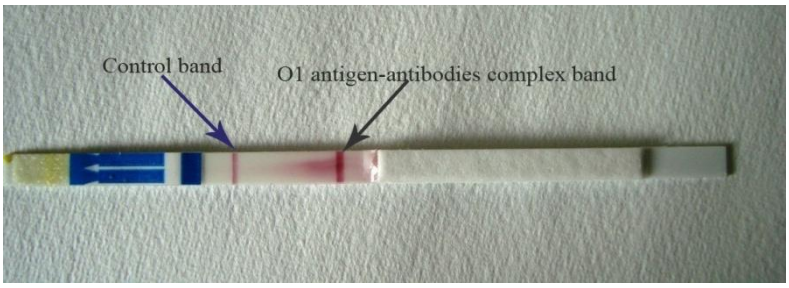

Fig.(1): *Vibrio cholerae* O1 positive result by immunochromatographic one-step visual test. Two bands of O1 antigen –antibodies complex and control are appear.

The results of *ctxA2-B* gene amplification showed that, 9 (90%) out of 10 O1serotypes was positive and only one (10%) was negative. While the only one 100% O139 serotype was positive Table (4), figure (2).

Table (4): results of *ctxA2-B* gene amplification

Results	O1	O139	Total
Positive (%)	9(90%)	1(100%)	10 (90.9%)
Negative (%)	1(10%)	0(0%)	1(9.1%)
Total	10(100%)	1(100%)	11(100%)

Discussion

The present study investigate the incidence of *V. cholerae* serotypes in diarrheal cases of children, 25% of the cases was due to *V. cholerae* O1 and O139. It was high percentage as there are many diarrhea

causative agents in children [18].

All of serotypes obtained were belonging *V. cholerae* O1 in the outbreak in Iraq in 2007–2009[19]. In this study, O139 serotype was found in outbreak of 2011.

Vibrio cholerae serotype O1 had been considered the only causative agent of epidemic cholera until the emergence of *V. cholerae* serotype O139 in Bengal in 1992 in southern India [20**]** .The first incidence of O139 was recorded in Baghdad, Iraq, in 1999 [21]. This study is the second incidence of O139 serotype. *V. cholerae* O139 can disseminate widely, causing severe watery diarrhea that is clinically indistinguishable from that caused by *V. cholerae* O1 strains.[20]

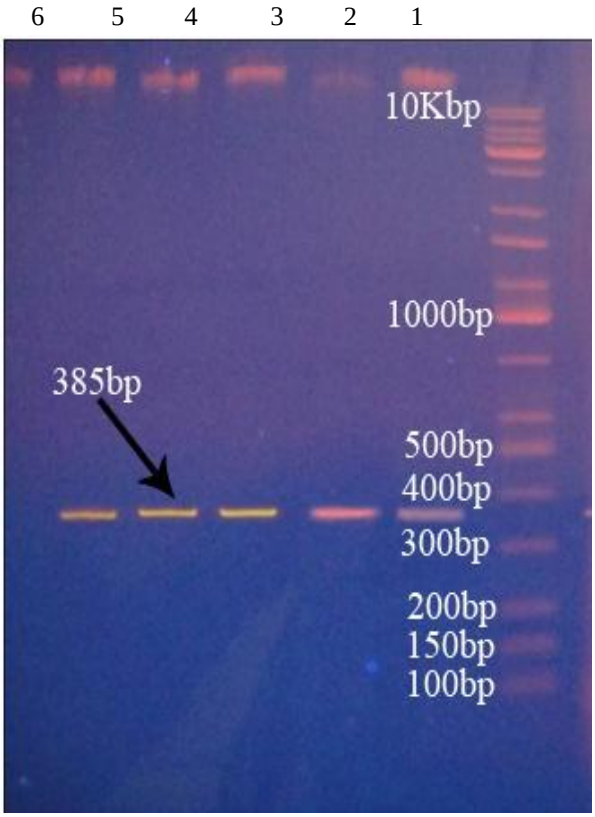

Fig. (2): Agarose gel electrophoresis of *ctxA2-B* gene PCR products. Lane 1:100bp ladder, lanes 2-6 represent *ctxA2-B* PCR products of *Vibrio cholera* (385 bp). Electrophoresis was carried out in 1.5% agarose gel supplied with Ethidium bromide at (7V/cm) for 90 minutes.

In our study 90% of O1 serotype was positive to *ctxA2-B* gene this result is relatively similar to that of [6] who found that 91.7% of O1 serotype was positive ,while *l* [10], and [22] found that 100% of O1 serotype was positive to this gene.

For the toxigenic strains of *V. cholerae*, there is a prophage known as CTX Φ integrated in the chromosome; this genetic element comprises a 4.5-kb central core region that contains *ctxAB* gene as well as, *zot*, *ace*, *orfU*, and *cep* genes, flanked by one or more copies of the repetitive sequence [23]. *ctxAB* gene can occur in multi copies in O139 serotype, since [24] found that, 68.8% of O139 serotype had two copies of *ctxAB*, 23.9% had one copy and 7.3% had three copies or more. The high bacterial toxigenic potential may be attributed to the high copy number of cholera toxin gene.

The rapid test, like Immunochromatographic one step visual test, in combination with effective health management would result in lower incidence of mortalities during the culture period. On the other hand rapid molecular methods, like PCR, can be used as a rapid method for detection of pathogenic *Vibrio* spp [13] and can give an idea about their toxigenic potential and their ability to cause severe disease [17].

We conclude that, *V. cholerae* O1 is more predominant than *V. cholerae* O139 among *V. cholerae* strains isolated from cases of cholera in Baghdad. Immunochromatographic test is a rapid and sensitive method in recover *V. cholerae* serotypes. Cholera toxin gene, *ctxA2-B*, is a common gene among both *V.*

cholerae O1 and O139 serotypes in the isolates of this study. PCR is a simple molecular tool to determine the toxigenicity of *V. cholerae* isolates.

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