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Rapid Screening of toxigenic and non-toxigenic *V. cholera* from clinical and environmental samples in Iraq by PCR

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Abstract:

Zonnla occludens toxin (zot) is one of the major virulence factors of *Vibrio cholera*. The detection of zot producing *V. cholerae* using conventional culture, biochemical and immunological based assays is time-consuming laborious, and requiring more than three days to perform. In this work aspecific primers for zot gene were used for detection of *V. cholerae* that isolated from clinical and environmental samples.

The results revealed that 12 of 15 clinical isolates were Ol serogroup, 6 isolates belonged to each of serotypes ogawa and lnaba and 3 isolates belonged to the serotype Non/ Ol, and all environmental isolates (5 isolates) were belonged to the serotype Non/ O1. Few colonies of *V. cholerae* were suspended in nutrient broth and used as few plates in PCR reaction for the detection of zot gene, The 947 bp sequence of a gene that codes for the zonula occludens toxin was amplified by PCR. Direct use of *V. cholerae* pure culture for PCR replaces the need for DNA extraction or boiling.

PCR results showed that all clinical (Ol) isolates of *V. cholerae* contain gene namely zot, while the Non/ O1 isolates that had been isolated from clinical sources, as well as, that isolated from environment showed no existence for zot gene.

The results of Molecular epidemiology study indicated that the source of outbreaks of 2007, 2008 and 2009 is the same all over Iraq, according to the PCR profile.

Key words: toxigenic, non-toxigenic, pcr, *V. cholera*

الخلاصة:

يعتبر الذيفان المعوي (zot) من اهم عوامل الضراوة لبكتريا الكوليرا المسببة للإسهال المائي، ولأن الكشف الروتيني عن بكتريا الكوليرا المنتجة للذيفان والمتضمن زراعة البكتريا على الوسط الزرعي (TCBs) واجراء الاختبارات المناعية والكيموحيوية تستهلك جهداً ووقتاً يتعد ثلاثة ايام. تم في هذا البحث استخدام بادئات خاصة لجين zot لغرض الكشف عن بكتريا الكوليرا المعزولة من مصادر سريرية وبيئية.

اظهرت نتائج هذه الدراسة بأن هناك 12 عزلة من مجموع 15 عذلة سريرية تعود الى المجموعة المصلية Ol، 6 عزلات منها تعود الى كل من النمطين المصليين lnaba وOgawa و3 عزلات تعود الى النمط المصلي Non/ O1، كما بينت الدراسة ايضاً بأن جميع العزلات البيئية الخمسة تعود الى النمط المصلي Non/ O1. تم تعليق عدد من مستعمرات الكوليرا في المرق المغذي لغرض استخدامها كقالب في فحص التفاعل التسلسلي المتضاعف لمعرفة امكانية استخدام هذا الفحص بالكشف عن وجود الجين zot. لقد تم تضخيم تسلسل معين من الجين المسؤول عن انتاج الذيفان والذي يبلغ حجمه 947 زوج قاعدة. ان استخدام الخلايا البكتيرية النقية مباشرة بالفحص قد اغنى عن استخلاص الدنا او الغليان.

أظهرت الـ PCR بأن جميع العزلات السريرية العائدة للنمط المصلي 01 تحتوي على الجين (zot)، بينما جميع العزلات السريرية والبيئية التي تعود الى النمط المصلي Non/ O1 لا تحتوي على الجين، أثبتت الدراسة الوبائية الجزئية لضمات الكوليرا في العراق بأن جميع عزلات البكتريا المسببة للأوبئة خلال السنوات الماضية 2007-2009 تعود لنفس المصدر وفقاً لنتائج الـ PCR.

مفتاح الكلمات: السام وغير السام وضمات الكوليرا و PCR.

Introduction:

Cholera is an acute intestinal infection caused by the bacterium *Vibrio cholerae*, which produces an enterotoxin (cholera toxin) that causes severe diarrhea which characterized by capious, painless, watery stool, often accompanied by vomiting that can quickely lead to severe dehydration and death if treatment is not promptly given[1,2].

Other toxins may be produced by toxigenic *V.cholerae* contributing to its pathogenicity as zonula occludens toxin (zot) which increases the permeability of small in testinal mucosa by affecting the structure of the intercellular tight junction or zonula occludens [3].

The gene that encodes the zot toxin is found on each chromosomes of *V. cholerae*[4,5].

The water borne pathogen *V.cholerae* is the causative agent of epidemics of cholera in many developing countries including Iraq, *V.cholerae* is a well Known human pathogen causing cholera epidemics worldwide, of at least 206 known serogroups of *V.cholerae*, only two serogroups O1 and O139 strains are responsible for epidemic outbreak of cholera [6].

There is a need for rapid identification of this Pathogen in order to prevent disease caused by exposure to contaminated water and food sources, and the other goals of this research is to be able to figure out the epidemiological source of *V.cholerae* that participated in outbreaks periods of 2007, 2008 and 2009, to do so, simplex PCR has been used to a ccomplished this goal and to diagnosis toxigenic *V. cholerae* by detection of zot gene.

Material and Methods:

Bacterial isolates: all clinic al *V. cholerae* isolates were obtained from central public Health Laboratory and that collected from Baghdad and different provinces in Iraq during outbreaks 2007-2009, and all environmental isolates were isolated from different water rivers in Baghdad and other provinces (karbala, Basrah, sulaymaniyah, wasit and Muthana) during a period from may 2009 to November 2009. Clinical and environmental *V. cholerae* isolates that used in this study was diagnosed in the central public Health Laboratory .*V. cholerae* cultured on thiosulfate citrate bile salt sucrose (TCBs) agar and incubated at 37C^{o} for 18-24 hrs.

PCR assay: specific primers selected for PCR analysis of zot gene (Gene bank accession) are shown in table (1) according to [7, 8]. These primers synthesized by Alpha company, Canada.

PCR reaction was conducted in 50 µl of a reaction mixture containing 10 µl suspension of few bacterial colonies, 24 µl go tag Green Master, 2 µl Mgcl2, 2 µl of each primer and 10 ml distilled water. Amplification was conducted using a master cycler (eppendrof) programmed with 1 cycle at 95C^{o} for 1 min, 40 cycle of 95 C^{o} for l min. 66 C^{o} for 1 min, 72 C^{o} for 1 min, 72 C^{o} for 10 min, the amplified product was subjected to 1.8 agarose gel electrophoreis and visualized under UV (Image master VDS, pharmacia Biotech USA) after ethidium bromide staining.

Results and discussion:

The core of present study is to prove the ancestory of *V. cholerae* isolates during different outbreaks and different times, to do that, simplex PCR has been chosen to be the vehicle to accomplish this goal. Although, the DNA must be released from the bacterial cells by different methods, such as biochemical DNA extraction and boiling for 10 min [10].

Table-1: Sequence and concentration of forward and reverse primers used for the amplification of *zot* gene in toxigenic *V.cholerae*.

Primer type	Primer sequence	Concentration In picomol	Product size
Forward primer *ace- F*	TCGCTTAACGATGGC GCGTTTT	144573	947bp fragment
Reverse primer *ace-* R	AACCCCGTTTCACTTC TACCCA	133919	

Simplex PCR assay was carried out, by using direct *V. cholerae* isolates without DNA extraction or boiling instead a pre-denaturation step was used for 5 min at 95^{o}C[9].

In order to optimize the optimum annealing temperature a gradient PCR was used, the results showed that 66-70 C^{o} optimum for amplification the target sequence with a sharp band (figure -1). It was observed on a 1.8% agarose gel. The fragment size was 947 bp for zot gene as shown in figure -1.

The results presented in figure- 1 gave indication that all clinical isolates O1 were positive for possessing zot gene while all clinical and environmental Non/ O1 isolates gave negative results for the existence of zot gene, The results of current study were identical to the results of some studies [8,11].

It was demonstrated, that is a good chance for *V. cholerae* of environmental origin to play an important role in spreading of these vibrio which causes the diseases and outbreaks, since they provide as an environmental reservoirs for clinical isolates that could be spreading from them to human and cause disease [12].

After *V. cholerae* releasing from human to the environment undergo some physical and genetical changes, most of them termed to produce L-form shape due to adrastic change in cell wall component [13,14]. As a result, the location of the product of zot gene will be affected, consequently, the expression of zote gene will be stopped that because there is no location any more for this product of this gene, (as a defence mechanism For bacteria as it is exited in abnormal environment, it turned to save energy for its life) [15], since the location of the production of the gene is a periplasmic space, then upon the deformity of the cell wall then, it can be expected that periplasmic space will be altered too, that may explain the absence of this gene from Non/ O1 clinical and environmental isolates, this will leads us to conclude that all environmental isolates were clinical isolates in origin , underwent some genetic changes or deformities that give it the character of environmental isolates , or a dramatic changes in the cell component may take place and that could affect the capability of such isolates to react positively with the antisera of *V. cholerae*, that will lead us to hypothysis that the NAG bacteria were once a clinical isolates.

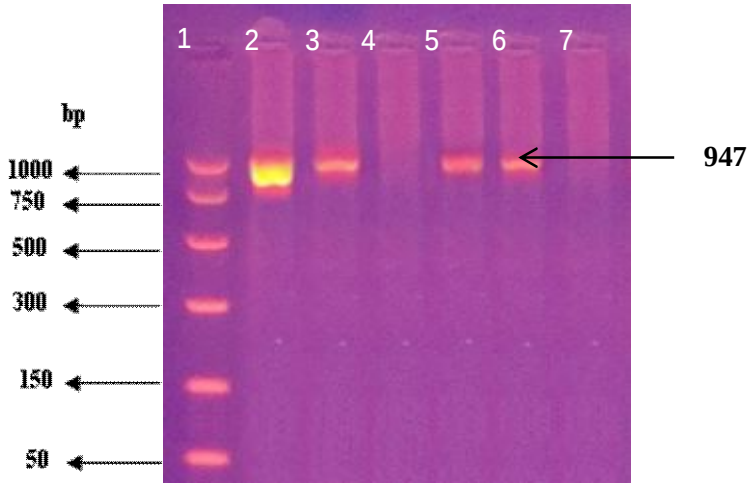

Figure 1: Agaros gel electrophoresis (1.8%) of PCR amplicon of *zot* gene of *V. cholerae*isolates for 1.5 hr at 5 volt/cm.

Lane 1: Lambda ladder Marker (1000 bp)
Lane 2: *V. cholerae* (Inaba) Isolated from Sulaymaniyah province during 2007 outbreak.
Lane 3: *V. cholerae* (Inaba) Isolated from Basrah province during 2009 outbreak.
Lane 4: *V. cholerae* (Non /O1) (Environmental source)
Lane 5: *V. cholerae* (Ogawa) Isolated from Wasit province during 2008 outbreak.
Lane 6: *V. cholerae* (Ogawa) Isolated from Baghdad province during 2009 outbreak
Lane 7: *V. cholerae* (Non /O1) (Clinical Source)

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