Detection of toxigenic *Vibrio cholerae* by PCR

الكشف عن بكتريا ضمات الكوليرا المنتجة للسموم باستخدام الـPCR

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Abstract

Cholera toxin (CT) is a major virulence factor of *V. cholerae* causing water diarrhea. The detection of CT-producing *V. cholerae* using conventional culture-, biochemical- and immunological-based assays is time-consuming, laborious, and requiring more than three days perform. In this work a specific primers for *ctx*B gene were used for detection of *V. cholera* in water samples. Few colonies of *V. cholera* were suspended in water and used as a template in PCR reaction for the detection of *ctx*B gene. The 391-bp sequence of a gene that codes for the cholera toxin B subunit was amplified by PCR. Direct use of *V. cholerae* pure culture for PCR replaces the need for DNA extraction or boiling. Increase the concentration of $MgCl_2$ enhances the efficiency of amplification. The specificity of the assay was determined to be specific for *V. cholerae* but not for, vibrio related bacteria, *E. coli*, Non-Agglutinable (NAG) *V. cholerae*, and *Aeromonas* sp.

المستخلص

يعتبر ذيفان الكوليرا من اهم عوامل الضراوة لبكتريا الكوليرا المسببة للاسهال المائي . ولان الكشف الروتيني عن بكتريا الكوليرا المنتجة للذيفان والمتضمن زراعة البكتريا على الوسط الزرعي واجراء الاختبارات المناعية والكيموحيوية تستهلك جهدا ووقتا يتعدى ثلاثة ايام . تم في هذا البحث تصميم بادئات خاصة لجين *ctx*B لغرض الكشف عن بكتريا الكوليرا في النماذج المائية . علقت عدد من مستعمرات بكتريا الكوليرا في الماء لغرض استخدامها في فحص التفاعل السلسلي المتضاعف لمعرفة امكانية استخدام هذا الفحص في الكشف عن وجود الجين *ctx*B . وتم تضخيم تسلسل ضمن الجين المسؤول عن جزء من الذيفان وهو بيتا والذي يبلغ حجمه 391 زوج قاعدي . ان استخدام الخلايا البكتيرية النقية مباشرة بالفحص قد اغنى عن استخلاص الدنا او الغليان . لوحظ ان اضافة كمية اضافية من الملح $MgCl_2$ حسن من اداء الفحص . تم التاكد من خصوصية الفحص من خلال اعطائه نتيجة موجبة لبكتريا الكوليرا المنتجة للذيفان وسالبة لبكتريا قريبة من بكتريا الكوليرا وهي بكتريا القولون وبكتريا الكوليرا غير المنتجة للذيفان وبكتريا الايروموناس .

Introduction

Waterborne pathogenic microorganisms, such as those found in ground and surface waters, pose a major risk to human health. *Vibrio cholerae* is a well-known human pathogen causing cholera epidemics worldwide [1]. It is the causative agent of epidemics of cholera in many developing countries including Iraq. Of at least 206 known serogroups of *V. cholerae*, only two serogroups O1 and O139 strains are responsible for epidemic outbreak of cholera [1].

Rapid and accurate identification of *V. cholerae* in the potable water and aquatic environment is important for disease management and public health protection. The

conventional culture method, which is routinely used for the isolation of *V. cholerae*, is time-consuming and may not be suitable for cells that are VBNC [2 , 3]. Recent molecular techniques such as polymerase chain reaction (PCR) offer both a rapid and reliable method for detecting toxigenic *V. cholerae* occurring in the aquatic environment [4 , 5].

The bacterium secretes cholera toxin that is responsible for the profuse watery diarrhea [6]. The toxin, an 85kDa protein, comprises of one cholera toxin A (CTA) and five B subunits (CTB) combined in an AB5 holotoxin. CTA is composed of two polypeptides (22ka, 5kD) responsible for the toxic activity of the toxin [7]. The B subunit pentamers (55.6kDa) are nontoxic and responsible for the binding of the toxin to ganglioside M1 (GM1) receptors present on the surface of intestinal epithelial cells [8]. There is a need for rapid identification of these pathogens in order to prevent disease caused by exposure to contaminated water sources. The aim of this study is to diagnose toxigenic *V. cholera* using PCR for *ctx*B gene.

Materials and methods

Bacteria: *V. cholerae*, and other bacteria used in this study was diagnosed in the central health laboratory. *V. cholera*e cultured onto thiosulfate-citrate-bile-salt-sucrose (TCBS) agar and incubated at 37°C for 18 to 24 h. Other bacteria, *Escherecia coli*, *Aeromonas* sp, and NAD *V. cholerae* were cultured overnight onto nutrient agar medium at 37°C.

PCR assays. Specific primers designed for PCR analysis of *ctxB* gene (Gene bank accession number AF390572) are shown in Table 1. These primers synthesized by Alpha DNA Company, Canada.

PCR reaction was conducted in 50 µl of a reaction mixture containing 11µl suspension of few bacterial colonies, 25 µl **GoTaq® Green** Master (Promega, CA), (1.5 µl) 25mM $MgCl_2$, 400 Pmol of each primer, 3µl of distilled water. Amplification was conducted using a Mastercycler (Eppendorf) programmed with 1 cycle at 95°C for 1 min; 40 cycles of 95°C for 1min, 56°C for 1min, 72°C for 1min; 72°C for 10min. The amplified product was subjected to 1.5% agarose gel electrophoresis, and visualized under UV (Imagemaster VDS, Pharmacia Biotech, USA) after ethidium bromide staining.

Table (1): Primers used for the amplification of *ctxB* in toxigenic *V. cholerae*.

Primers	Primer sequence (5_–3_)	Nucleotide position	Amplicon size (bp)
Forward	**TGAATTATGATTAAATTAAAATTTG**	**941–967**	**391**
Reverse	**TTTATATCTTAATTTGCCATACTAA**	**1306–1331**	

Results and discussion

For amplification of *ctx*B gene, the DNA must be released from the bacterial cells by different methods, such as biochemical DNA extraction [12] and boiling for 10min [9]. In this work direct PCR for *V. cholera* was used without DNA extraction or boiling instead a pre-denaturation step was used for 5min at 95°C [6].

In order to optimize the optimum annealing temperature a gradient PCR was used, the results showed that 55-57°C optimums for amplification the target sequence but with a faint band. In order to improve efficiency of PCR, 1 to 5µl of 25mM $MgCl_2$

concentrations were added, in addition to the initial concentration of $MgCl_2$, which was 1.5mM/50µl reaction. A sharp band was detected on agarose gel electrophoresis with 4 µl of 25mM $MgCl_2$ and specific and nonspecific bands were detected with 5µl of 25mM $MgCl_2$, Figure (1). Increasing the concentration of $MgCl_2$ in PCR reactions tends to promote binding of primers and adding $MgCl_2$ can prove useful in cases where no amplification products are observed. If the concentrations is too high, however nonspecific primer binding can occur resulting in amplifications that are multi-banded or smeared when visualized on an agarose gel [10].

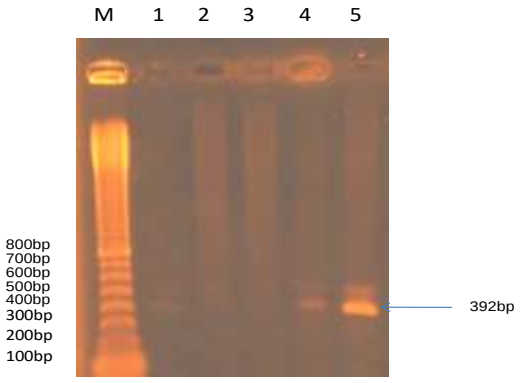
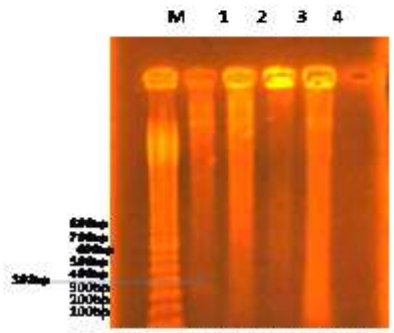


Figure 1: Agarose gel electrophoresis (1.5%) of PCR reaction for ctxB gene of *V. cholera* (Inaba). M: 100bp DNA marker; Lane1: 1 µ l MgCl2; Lane2: 2 µ l MgCl2; Lane3: 3 µ l MgCl2; Lane 4: 4 µ l MgCl2; Lane 5: 5 µ l MgCl2.

The specificity of PCR for toxigenic *V. cholerae* was determined by doing PCR for other related bacteria Figure (2). *E coli* was used because it has B subunit toxin with 80% similarity to B subunit of *V. cholerae*, non toxigenic NAG *V. cholerae* was used to differentiate toxigenic from toxigenic *V. cholerae*, Aeromonas sp. was used because it is a water borne bacteria, and produce enterotoxin and produce **diarrhea** as *V. cholera*[11].



It was concluded that the specific primers in this study were specific and sensitive for detection of toxigenic *V. cholerae* from pure culture colonies using polymerase chain reaction without the need for DNA extraction. The finding of this study could be applied for testing water samples contaminated with toxigenic *V. cholerae* with high efficacy and few hours.

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