The use of *Omp W* Gene in Detection of *Vibrio cholerae* Isolated from Diarrhea Cases of Children in AL-Diwaniya Province.

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الخلاصة

تم خلال هذه الدراسه جمع 221 عينه براز من اطفال تحت سن الخامسه عشريعانون من اسهال مائي ومن كلا الجنسين والذين راجعوا مستشفى الولاده والاطفال في محافظه الديوانيه خلال الفتره الزمنيه من تشرين الاول 2008 وحتى حزيران2009. كذلك تم جمع 27 عينه ماء من ثلاثه مناطق مختلفه لنهر الديوانيه وبنفس الفتره الزمنيه لغرض دراسه الصفات المظهريه والجزيئيه لضمات الكوليرا وذلك لغرض تقييم طرق التشخيص المختبريه الروتينيه المستخدمه في تشخيص سلالات ضمات الكوليرا المختلفه ومقارنتها بتقنيه تفاعل السلسله المتبلمره (PCR).

تم عزل وتشخيص ضمات الكوليرا باستخدام طريقه الزرع اضافه الى الاختبارات الكيموحياتيه ونظام التشخيص (API 20E). كذلك اظهرت نتائج التنميط المصلي باستخدام Polyvalent *V. cholerae* O1 antisera و monovalent Ogawa و monovalent Inaba) ان اغلب ضمات الكولير المعزوله سريريا كانت تعود الى النمط المصلي (O1) بينما جميع ضمات الكوليرا التي عزلت من المياه السطحيه لنهر الديوانيه كانت تعود الى النمط المصلي (Non- O1).

تم استخدام فحص تفاعل السلسله المتبلمره (PCR) للكشف عن جين (*omp*W) الذي يشفر لبروتين الغشاء الخارجي الخاص بضمات الكوليرا. اعتمادا على نتائج فحص تفاعل البلمره، اظهرت هذه الدراسه ان معدل عزل ضمات الكوليرا كان 5.9% بالنسبه لعينات البراز بينما 14.8% بالنسبه لعينات الماء. كذلك اظهرت نتائج فحص تفاعل السلسله المتبلمره (PCR) ان هنالك خصوصيه عاليه (100% و100 % و 97% و86 %) في الكشف عن سلالات ضمات الكوليرا ضد كل من الطرق الروتينيه المختلفه كالزرع، الاختبارات الكيموحياتيه، نظام (API 20E) والتنميط المصلي على التوالي.

Abstract

A total of 221 stool samples were collected from children suffering from watery diarrhea, less than 15 years old of both genders whom admitted to the Maternity and Children Teaching Hospital in Al- Diwaniya Province, In addition to that, 27 water samples were also collected from three different loci of AL-Diwaniya River, at the period of October 2008 to June 2009,

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in order to evaluate the routine laboratory diagnostic procedures in the diagnosis the multi-serogroups or serotype of Vibrio cholerae strains and compared them with molecular technique as Polymerase chain reaction (PCR).

Vibrio cholerae has been isolated and identified by using culturing method in addition to biochemical tests, API 20E diagnostic kit. Serotyping by using polyvalent Vibrio cholerae, O1antisera, and monovalent Ogawa and Inaba revealed that the most of clinical V. cholerae were of serogroup O1, while all the V. cholerae isolated from surface water of AL- Diwanyia river was Non-O1 serogroup. PCR technique was used to detect ompW gene encoding to outer membrane protein of V. cholerae. Based on the PCR results, the rate of Vibrio cholerae isolation from stool samples was 5.9%. PCR results showed that there were high specificity (100%, 100%, 97% and 86%) in detection of Vibrio cholerae strains versus each of cultural, biochemical, API 20E system and serological tests, respectively.

Introduction

Cholera has been recognized as a killer disease since earliest time. The disease is caused by infection of the small intestine by Vibrio cholerae O1 and O1391 which is characterized by severe dehydrating diarrheal condition and is one disease in modern times that is epidemic, endemic and pandemic in nature2.

Cholera is a historically-feared epidemic of diarrheal disease that remains a major public health problem in many parts of Africa, Asia, and Latin America3. Though rare in developing countries, cholera is still an important infection worldwide4. Also cholera has been categorized as one of the "emerging and reemerging infections" 5 threatening many developing countries. Outbreaks of cholera cause deaths estimated at 120,000 annually worldwide and many more cases each year, of which the vast majority occurs in children6.

In the last decades, attention to cholera epidemiology increased, as cholera epidemics became a worldwide health problem. Detailed investigation of V. cholerae interactions with its host and with other organisms in the environment suggest that cholera dynamics are much more complex than previously times7. V. cholerae O1 and O139 are currently believed to be the only serogroups causing epidemic cholera8. The natural inhabitant of Vibrios is the aquatic environment and water plays an important role in disease transmission9.

In the past, the ability to differentiate V. cholerae strains for epidemiological purposes was hampered by the low discriminatory capability of available systems, such as biochemical and serologic identification10. Molecular techniques should be considered as potentially important tools in the effective monitoring of strains of V. cholerae especially those associated with epidemiologically well-defined outbreaks. So, application of molecular techniques to the analysis of strains has enhanced epidemiologic investigations and understanding of the sources and vehicles of transmission11.

Materials and Methods

1- Samples collection

A total of 221 stool samples were obtained from patients who attended to Maternity and Children Teaching Hospital in Al-Diwanyia province, during the period from October 2008 to the June 2009. Patients mainly included children under 15 years old from both genders suffering from watery diarrhea. The stool samples were collected in sterile plain plastic containers. Then, a small amount of samples was injected in to 5 ml of alkaline peptone water (APW) prepared earlier in sterile tubes. In addition to that, 27 water samples were also collected from three different loci of AL-Diwaniya River, by using the membrane filter technique, a 500-1000 ml of water samples were collected, concentrated on 0.22 µm Pore – diameter filters, followed by incubation of the membranes in an enrichment medium of alkaline peptone water (APW) over night at 37 ºC.12.

2- Samples processing

A loopful of the culture broth, was taken from the top layer of the APW then streaked on to Thiosulphate Citrate Bile salt Sucrose agar (TCBS) and incubated overnight at 37 ºC. Two-three yellow, flat, 1-3 mm diameter isolated colonies were picked from each sample and streaked on blood agar and MacConkey agar for further testing12, 13.

3- Isolates identification

The isolates were further identified by microscopic examination and biochemical tests13, 14. Also the isolates confirmed biochemically by using API 20-E system (Biomeriemx / france) according to instructions of supplied company.

4- Serotyping

Serologic confirmation was done using polyvalent, anti-Ogawa, and anti-Inaba antisera from Plasmatec Laboratory Products Ltd (Plasmatec/ UK)15.

5- Biotyping

The biotype of V. cholerae serogroup O1 strains was distinguished by the following methods; polymyxin B susceptibility, hemolysin test, and Voges-Proskauer test15.

6- Polymerase chain reaction (PCR) detection method

A-Method of DNA extraction: DNA extraction of isolated **colony of Vibrio cholerae was done according to DNA extraction protocol of manufacturing company instructions (Cinagen / Iran) (Figure 1).**

B- Method of PCR Reaction: The PCR amplification was **performed using assay based on repetitive sequence ompW gene, the primers forwared (5́-CAC CAA GAA GGT GAC TTT AAT TGT G-3´) and revers (5´-GAA CTT ATA ACC ACC GCG -3´),16. Also the laboratory protocol was done according to manufacturing company instructions (Labaqua / Spanish).**

Statistical analysis

The results were analyzed statistically by Chi-square (X^2) test at the level of significant when P-value < 0.01.The specificity, sensitivity and diagnostic accuracy of the results were also calculated by applying the following equations: a / a + b×100 = sensitivity, d / c+ d×100 = specificity.

a= Total of true positive, b= Total of false positive, c= Total of false negative and d= Total of true negative, positive and negative predictive value also calculated. Diagnostic accuracy = sensitivity + specificity / 2.17.

Results and discussion

This study shows that traditional typing systems based on serotype, biotype and other traditional methods are not always able to discriminate and identification these strains that isolated. Serotyping by using polyvalent cholera O1 antisera and monovalent Ogawa and Inaba revealed that the most of clinical V. cholerae were of serogroup O1, while all the V. cholerae isolated from surface water of AL- Diwanyia river was Non-O1 serogroup and all the

agglutinating strains in this study were identified as V. cholerae O1, biotype El Tor, serotype Ogawa.

This study showed that out of 221 stool samples and 27 water samples, (33) stool samples (14.9%) and (9) water samples (33.3%) were positive for culture method while 16 stool samples and 5 water samples gave positive by biochemical tests. On the other hand, 13 stool samples and 3 water samples showed positive results in API 20E diagnostic kit while only 12 stool samples gave positive result for serotying by polyvalent V. cholerae O1antisera. PCR technique showed only 13 stool samples and 4 water samples were positive.

The results of this study also revealed that all strands of DNA which resulted from the binding between specific primers and positive isolates extracted DNA appear as single band with 588 bp under the U.V light using ethidum bromide as a specific DNA stain in addition to band with the 650 pb work as Internal Positive Control (IPC) (Figure 2).

Comparison Between The PCR Method and Other Tests Used for Detection of *Vibrio cholera*e

a- PCR Results Versus Culture Method

From 48 samples were tested by culture and PCR, there were 42 samples positive by culture method versus 17 samples positive by PCR. So, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy rate of PCR were (40%, 100%, 100%, 19% and 70%) respectively as demonstrated in (Table 1).

b- PCR Results Versus Biochemical Tests

From 48 samples were tested by biochemical tests and PCR, there were 21 samples positive by biochemical tests versus 17 samples positive by PCR. So the sensitivity, specificity, PPV, NPV and accuracy rate of PCR were (81%, 100%, 100%, 87% and 90.5%) respectively (Table 2).

c- PCR Results Versus API 20E

From 48 samples were tested by API 20E and PCR, there were 16 samples positive by API 20E versus 17 samples positive by PCR. The sensitivity, specificit
y, PPV, NPV and accuracy rate of PCR were (100%, 97%, 94%, 100% and 98.5%) respectively (Table 3).

d- PCR Results Versus Serotyping

From 48 samples were tested by API 20E and PCR, there were 12 samples positive by serotyping versus17 samples positive by PCR. The sensitivity, specificity, PPV, NPV and accuracy rate of PCR were (100%, 86%, 70%, 100% and 93%) respectively (Table 4).

This study found that cholera infection in AL- Diwaniya provine considered one of the causes of diarrhea in children of age more than one years old. This may be reflected that Vibrio cholerae are one of etiological agent of diarrhea that infected human and the infections occurred in a regular seasonal pattern and are particularly associated with poverty and poor sanitation.

The traditional identification of V. cholerae is usually achieved through a series of biochemical tests after their growth and isolation on a selective plating medium e.g., TCBS agar18. Several studies conducted in Iraq revealed that the prevalence of Vibrio cholerae were (42. 9%), (47.14%), (87%) which reported by AL-Obidi, (2005); Kadhim,(2009); AL-Karkhy, (2005) respectively19, 20, 21.

This study revealed significant differences between culture method and PCR for diagnosis of Vibrio cholerae and the PCR more sensitive and specific than culture method because the culture method for Vibrio cholerae had very poor sensitivity and numerous false positive results was occurred22,23. Many bacteria can produce colonies on TCBS agar whose appearance is similar to the appearance of V. cholerae colonies and these false positive results take more time in their identification24.

According to the reading of biochemical tests, this study showed that from 42 isolate were positive by culture only 21 isolate was positive as Vibrio cholerae and the other 21 isolate were non Vibrio cholerae. This result was different with results of API 20E diagnostic kit which found that only 16 isolates were positive as V. cholerae. This study showed that there were no significant differences between PCR and biochemical tests and API 20E system in the diagnosis of Vibrio cholerae O1. The API 20E system is indeed considered an acceptable method for the identification of the more commonly-occurring members of the family Vibrionaceae25 even if there are very few reports expressly concerned with the ability of commercial systems to identify members of the genus Vibrio26. Baron et al., (2007) showed that the direct use of the PCR-based method for the definite identification of the screened colonies gave better results than the API 20E method16. Also, West et al. (1983) mentioned that the series of biochemical tests commonly used to identify V. cholerae was originally designed for clinical samples27.

In this study, serotyping of isolates by polyvalent O1 antisera we found 12 isolate belong to Vibrio cholerae O1 while the result of PCR were 17 isolate which belong to Vibrio cholerae. There were no significant differences between PCR and serotyping for diagnosis of Vibrio cholerae and we found PCR is more specific than serotyping in the diagnosis of Vibrio cholerae O1. Techniques based on serology are critical, but they are useful only for detecting specific serogroups. In contrast, the PCR-based identification techniques are accurate, sensitive, and permit a large throughput28.

Detection methods for Vibrio cholerae can be divided into two categories, conventional and rapid techniques. Conventional laboratory diagnosis depends upon isolation by culture and identification by biochemical and serological tests. This process, however, is time-consuming, take about 2 days, not always accurate because close relatedness among V. cholerae and certain other members of the Vibrio spp. or Aeromonas spp., with respect to their biochemical properties, has often made unambiguous identification of the organism quite difficult also it may be quite expensive for a laboratory28, 29. A rapid and accurate method is essential for the detection and screening of the public health threat V. cholerae. Hence, PCR method represents an assay format for the detection of V. cholerae and the DNA amplification technique is a rapid and very sensitive technique used22, 30, 31.

Table (1) Validity of PCR test for diagnosis of *V. cholerae* confirmed with culture method.

Test		PCR method		Total
		+ve	-ve	
Culture method	+ve	17	25	42
	-ve	0	6	6
Total		17	31	48

Table (2) Validity of PCR test for diagnosis of *V. cholerae* confirmed with biochemical tests.

Test		PCR method		Total
		+ve	-ve	
Biochemical tests	+ve	17	4	21
	-ve	0	27	27
Total		17	31	48

Table (3) Validity of PCR test for diagnosis of *V. cholerae* confirmed with API 20E.

Test		PCR method		Total
		+ve	-ve	
API 20E	+ve	16	0	16
	-ve	1	31	32
Total		17	31	48

Table (4) Validity of PCR test for diagnosis of *V. cholerae* confirmed with serotyping.

Test		PCR method		Total
		+ve	-ve	
serotyping	+ve	12	0	12
	-ve	5	31	36
Total		17	31	48

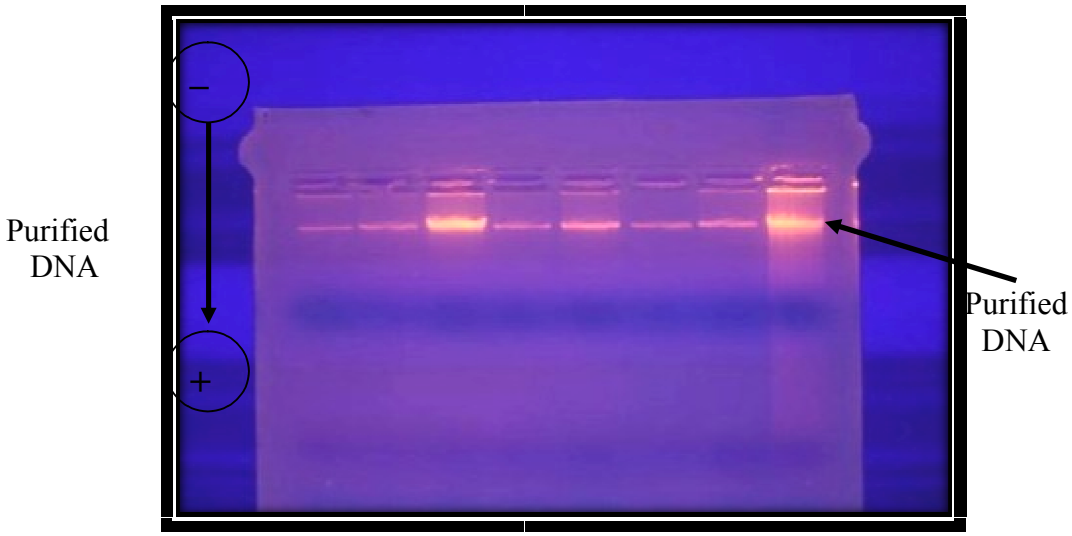

Figure 1: DNA extraction of *Vibrio cholerae* isolates using 1%agarose gel electrophoresis.

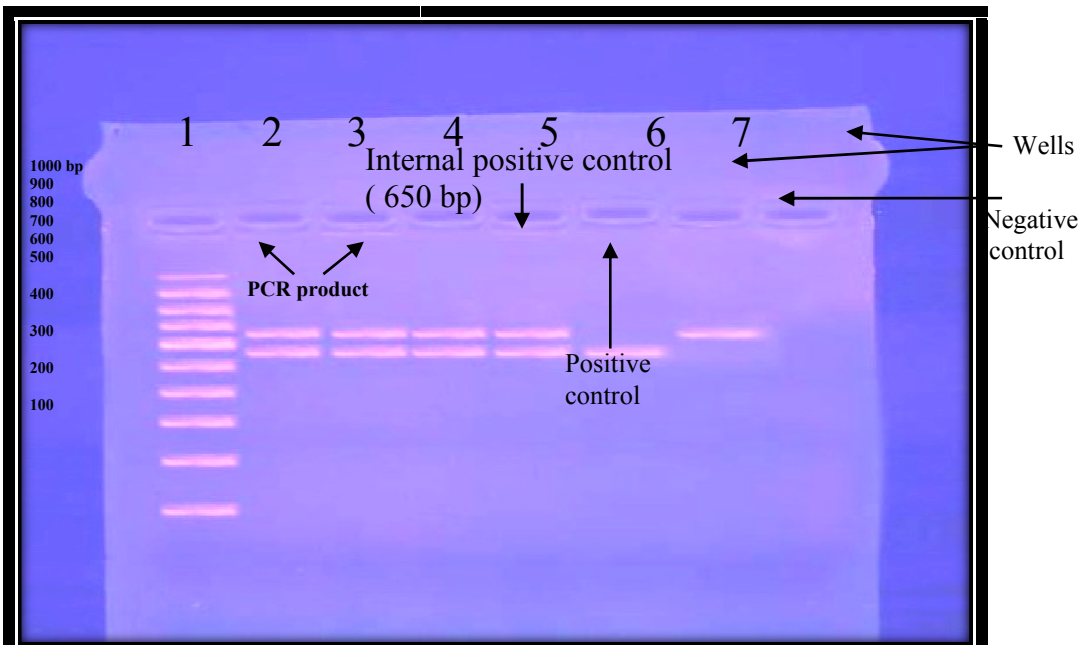

Figure 2: DNA amplification of a 588pb fragment of Omp W gene, Line 1, 1000 bp ladder , Line 2,3,4,5 positive specimens, line 6, positive control, line 7, negative control. Amplification samples was directly loaded in a 2% agarose gel containing 0.5 mg⁄ml ethidium bromide with adding loading buffer in electrophoresis.

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