Iraqi J. Vet. Med. 34 (2): 95 – 98; (2010).

Two Different Diagnosis Methods For The detection of cholera toxin production From Vibrio cholerae isolated from different areas in Iraq

Atheer Abdulrazzaq

College of veterinary University of Baghdad

Accepted – December – 2010

Summary

Fifty isolates of Vibrio cholerae obtained from different areas in Iraq from patients with acute secretory diarrhea were diagnosed serologically in central public health laboratory(CPHL), Two different methods were used for detection cholera toxin production ,the first based on the degradation of Nicotin Amide Adinine Dinucleotide(NAD) by Cholera toxin (CT). and the second was by detection CT gene by polymerase chain reaction (PCR).All Vibrio cholerae were cultured in alkaline peptone water, out of 50 isolates 46 (92%) were positive when we used NAD for the detection of cholera toxin(CT).Whereas all isolates gave positive results by PCR. From these data we conclude that the degradation of NAD by CT is simple and can be carried out in small laboratories, it is also easy to perform, and gives reproducible results, while PCR provides a more sensitive and specific assay for rapid diagnosis of cholera than currently available methods.

Key words:cholera, toxin,vibrio,.

طريقتان تشخيصية مختلفة للكشف عن سموم بكتريا الكوليرا المعزولة من مناطق مختلفة من العراق

اثير عبد الرزاق

كلية الطب البيطري جامعة بغداد

الخلاصة

خمسون عزلة من بكتريا الكوليرا تم الحصول عليها من مناطق مختلفة من العراق من مرضى يعانون من اسهال حاد تم تشخيصها سيرولوجيا في مختبر الصحة العامة المركزي. استعملت طريقتان مختلفتان للكشف عن سموم بكتريا الكوليرا , تميزت الطريقة الاولى بتكسير النيكوتين امايد ادنين داي نيكليوتايد بواسطة سموم بكتريا الكوليرا ,اما الطريقة الثانية كانت عن طريق الكشف عن الجين المؤول عن سموم بكتريا الكوليرا بطريقة التفاعل التضاعفي لسلسلة الدنا. جميع بكتريا الكوليرا زرعت على وسط ماء البيبتون القاعدي , اظهرت النتائج انه من بين 50 عزلة 46 (92%) كانت موجبة عندما استعملنا النيكوتين امايد ادنين داي نيكليوتايد لتكسير سموم بكتريا الكوليرا , بينما اعطت جميع العزلات نتيجة موجبة عندما استعملنا طريقة التفاعل التضاعفي لسلسلة الدنا . من هذه النتائج نستنتج ان طريقة تكسير النيكوتين امايد ادنين داي نيكليوتايد لسموم بكتريا الكوليرا هي بسيطة وسهلة الاستعمال ويمكن العمل بها في المختبرات الصغيرة اضافة الى كونها حساسة وتعطي نتائج جيدة. اما طريقة التفاعل التضاعفي لسلسلة الدنا كانت ذات حساسية ونوعية عالية للكشف عن الجين المسؤول عن سموم بكتريا الكوليرا مقارنة بالطرق التشخيصية التقليدية الاخرى.

Introduction

Vibrio cholerae is a waterborne pathogen that causes gastrointestinal disorder with a wide range of clinical manifestation, including vomiting and rice-like diarrhea (1). Various methods have been described for the detection of CT production in immunological assays such as ELISA(2),tissue culture assays(3) and polymerase chain reaction (PCR)(4).In our study we used a modified method for screening of CT production based on the principle that CT degrades nicotin amide adinine dinucleotide(NAD)(5). PCR method based on amplification of

target sequence of the CT gene has been reported by several workers(6).Polymerase chain reaction (PCR) was subsequently provide a more sensitive and specific diagnosis of cholera. A set of oligonucleotide primers were developed, and amplification condition for PCR to detect the CT gene were optimized, the detection limit of the PCR was determined to be 1pg of chromosomal DNA or broth culture containing three viable cells(7).

Material and Methods

Fifty isolates of Vibrio cholerae obtained from different areas Such as Abo Graib, south of Baghdad , Yousifia, Mahmoodia and Mosaib from patients with ages ranged between 6-15years suffering from acute gastroenteritis were studied for CT production. The stool specimens from diarrheal patients collected in sterile containers were transferred into 50 ml enrichment broth and incubated at 37ºC for 12 h. a loopful of growth was streaked in thiosulfate citrate-bile salt- sucrose agar plates and incubated at 37ºC for 18 h. Controls: CT toxin producing Vibrio cholerae (Oxoid) used as a positive control and Escherichia coli isolate tested to be non toxigenic was used as a negative control ,PBS control, Brain Heart Infusion broth (oxoid), and NAD were also included with each batch test as a negative control. **Procedure**: Test isolates were inoculated into alkaline peptone water and incubated for 18h at 37ºC. The turbidity of culture was adjusted to Mackfarland No1 using sterile saline(8).100ul 0f each broth were transferred to wells of a 96 well microtiter plate. 100ul of fresh NAD solution obtained from Randox chemicals(UK) was added to each well,the contents mixed and the plate incubated at 37◦Cfor 2h.100ul of 5N NaOH was then added to each well, the contents mixed and the plate incubated at room temperature in the dark for 1h The reading was taken using a lamp as UV light source. **Preparation of samples for direct PCR**: Preparation of genomic DNA. 10ml of broth culture was used for DNA extraction cells harvested at 14000 rpm for 5 min and resuspended in 200ul from TRI reagent(Acid Guanidinium Thiocyanate phenol-chloroform), the mixture was incubated at 65ºC for 15min, followed by sonication 3 times for 15sec,then added 200ul of triple distilled water to dilute the samples and added 400ul (phenol chloroform isopropanol)and mix the samples,then added 1000ul from cooled Ethanol 100% and mix the DNA samples by vortex, and then centrifuged at 14000rpm for 5min ,then the supernatant was discarded and added to the sediment 1ml from Ethanol 70%for washing ,then centrifuged at 14000rpm for 5 min,then discarded the supernatant and let the samples to dry at 37C,then diluted the DNA by 200ul distilled water .the concentration and purity of the DNA was then determined spectrophotometrically at A260 and A280. PCR primers and DNA amplification. A 22-bp forward primer(5ˉCGG GCA GAT TCT AGA CCT CCT G 3ˉ) and reverse primer (5ˉ CGA TGA TCT TGG AGC ATT CCC AC 3 ˉ)(6). The PCR assay was performed in a final reaction volum of 25ul. Each reaction mixture consisted of 0.3uM of each primer, 200um of each dNTp, 190uM dTTP,10uM digoxigenin-11-dUTP, 0.5U of Taq DNA polymerase (Sigma,USA),5 ul of 10x PCR buffer, 1.5mM $MgCl^2$ in the presence of genomic DNA and lysed cells. The PCR program was carried out at 94◦C for minutes fallowed by 5 and 30 cycles at 94◦C, 60◦C and 72◦C each for 45 sec, and the final extension at 72◦C for 5 minutes. Each PCR product (5ul) was loaded into a well of a 2% agarose gel containing 0.5 ug/ml ethidium bromide. A 100-bp ladder(sigma,USA) was used as the molecular weight marker. PCR products were electrophoresed and visualized under UV light, and gel images were stored using a gel documentation sydtem(Gel Doc 1000,Bio Rad,CA).

Results

Results showed that out of 50 isolates 46 (92%) were positive when we used NAD for detection cholera toxins and 4 (8%) gave negative result the positive results gave colorless reaction when we put the microtiter plate under UV light source and that's indicated the degradation of NAD by CT, whereas negative results gave fluoresces reaction when viewed under a UV light source which indicated intact NAD. PCR results. All isolates of V.cholera gave positive results obtained from PCR of genomic DNA and bacterial cells samples with 5 and 30 cycles were shown in figure 1. the minimum amount of genomic DNA and bacterial cell of V.cholera that produced an observable band on ethidium bromide –stained agarose gel electrophoresis (AGE) was 55pg of DNA with 30 cycles .

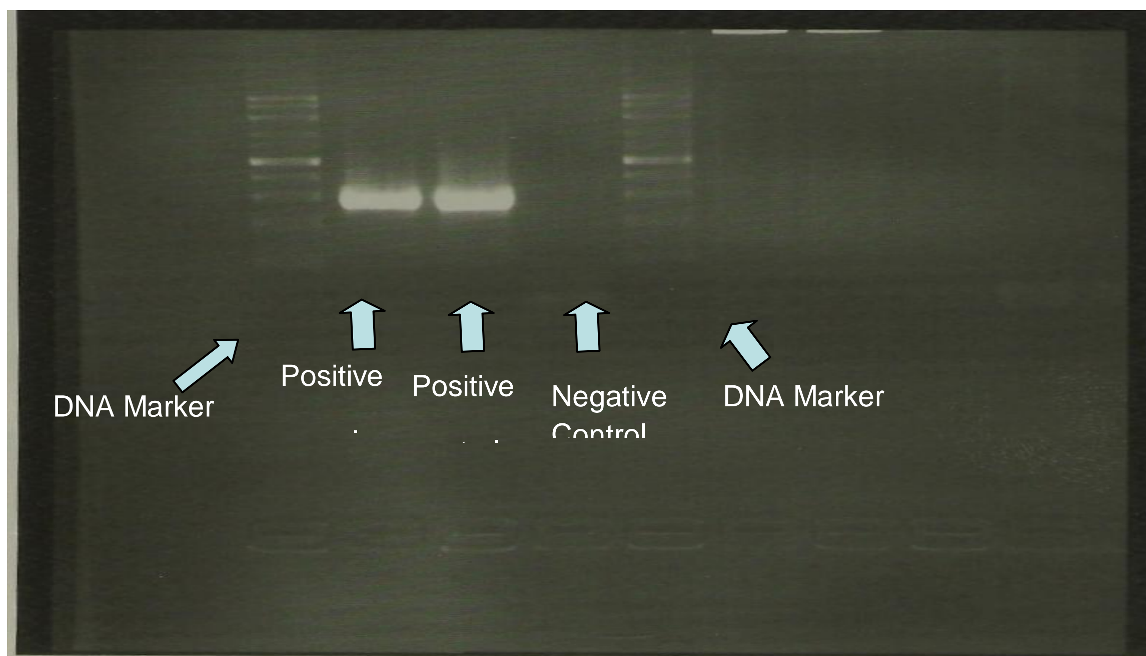

Fig. 1. sensitivity of PCR using cell lysate, followed by agarose gel electrophoresis. Lane 1 DNA Marker (100bp), Lane 2 Positive sample, Lane 3 Positive control, Lane 4 Negative control, Lane 5 DNA Marker

Discussion

There is a requirement for rapid, accurate measurements of Vibiro cholerae toxin which causes gastrointestinal disorder. In current study we used two different methods in diagnosis of cholera toxin (CT), the first one was used according to Osawa (9) with some modification for detecting intact/degraded NAD by cholera toxin makes the procedure simple and can be carried out in most laboratory testing vibrios for cholera toxin production, it is also suitable for large scale screening as many strains can be tested in a 96 well micro titer plate, including positive and negative control. The second one was PCR assay based on amplification of target DNA sequences in the CT gene of V.cholera have been reported (10). These investigators used PCR to identify toxigenic V.cholera in stool specimens, the specificity of the PCR was determined using 50 isolates collected from different areas from Iraq compared with E.coli, Pseudomonas and other toxin producers as negative controls, among all the bacterial isolates tested V.cholera was positive by PCR. Our data indicate a good correlation between intact / degraded NAD assay and PCR assay because of the similarity of results of the two methods. The use of PCR

is a sensitive and quantitative method that is useful for estimating the number of cells of a specific pathogen in a food product(11). The main advantage of PCR method is that it is very rapid and is a valuable method for screening a large number of samples. **In conclusion**, a rapid and accurate methods are essential for the detection and screening of the public health threat posed by toxigenic V.cholera. From these data we conclude that the degradation of NAD by CT is simple and can be carried out in small laboratories, it is also easy to perform, and gives reproducible results, while PCR provides a more sensitive and specific assay for rapid diagnosis of cholera than currently available methods.

References

1. Pourshafie MRF Grimont MS and Grimont PA (2000). Molecular epidemiological study of Vibiro choerae isolated from infected patients in Teheran, Iran J Med Microbiol. 49:1085-1090.
2. Ramamurthy T Bhattacharya SK Uesaka Y Horigome K Paul M,Sen D (1992). Evaluation of the bead enzyme- linked immunosorbent assay for detection of cholera toxin directly from stool specimens. J Clin Microbiol 28: 128-30.
3. De SN(1959). Enterotoxicity of Bacteria – free culture filtrate of vibirio cholera. Nature 183: 1533-4 .
4. Karunasagar IG Sugumar I Karunasagar and Reilley A(1995). Rapid detection of vibiro cholerae contamination of seafood by polymerase chain reaction. Mol Mar Biol Biotechnol. 4: 365-368.
5. Osawa R Okitsu T Sata S Yamai S (1997). Rapid screening method for identification of cholera toxin producing vibiro cholerae o 1 and o 139. J Clin Microbiol 35: 951-3.
6. Chirai HM Nishibuchi T Ramamurthy SK Bhattacharya SC pal and Y Takeda. (1991). Polymerase chain reactionfor detection of cholera enterotoxin operon of vibiro cholerae. J Clin Microbiol. 29:2517-2521.
7. Fields, PI Popvic T Watchsmuth K and Olsvik O(1992). Use of polymerase chain reaction for detection of Toxigenic vibrio cholera O1 strains from the latin American cholera epidemic. J Clin Microbiol. 30:2118-2121.
8. Osawa R Okitsu T Sata S Yamai S(199). Rapid screening method for identification of cholera toxin producing Vibiro cholerae o 1 and o 139. J Clin Microbiol 35: 951-3.
9. Jesudason Mary V(2000). A simplified procedure for rapid screening of vibirios for cholera toxin production . Indian J Med Res III, January 1-2.
10. Koch WHWL Payne Wentz BA and Cebula TA (1993). Rapid polymerase chain reaction method for detection of vibrio cholerae in foods .Appl Environ Microbiol. 59: 556-560 .

11.Lyon.J W (2001).Taq Man PCR for detection of Vibrio cholerae 01,O139,Non-O1 , and non O139 in pure cultures, Raw Oysters,and synthetic