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

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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.

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 adenine 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 6-15 years 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 37°C for 12 h, a loopful of growth was streaked in thiosulfate citrate-bile salt-sucrose agar plates and incubated at 37°C 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 37°C. The turbidity of culture was adjusted to Mackfarland No1 using sterile saline(8). 100ul of 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°C for 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 65°C for 15 min, 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 of cooled Ethanol 100% and mix the DNA samples by vortex, and then centrifuged at 14000rpm for 5 min, 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 37°C, 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 (5’ CGG GCA GAT TCT AGA CCT CCT G 3’) and reverse primer (5’ CGA TGA TCT TGG AGC ATT CCC AC 3’) (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 MgCl₂ in the presence of genomic DNA and lysed cells. The PCR program was carried out at 94°C for minutes followed 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 sytem(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.

Discussion

There is a requirement for rapid, accurate measurements of Vibrio 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

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