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Evaluation of Cholera Toxin Expression in Acidic, Alkaline and Neutral Conditions

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Background: Cholera is a severe disease which is caused by *Vibrio cholerae* and it is typically transmitted by either contaminated food or water particularly in developing countries. The most important virulence factor of this bacterium is an enterotoxin called cholera toxin which is a protein complex secreted by the *Vibrio cholerae*.
Objectives: In this project, we determined the production of cholera toxin at different pH values.
Materials and Methods: Two standard strain of Vibrio cholerae O_1 biovar EL Tor N16961 and Vibrio cholerae O_1 biovar Classic ATCC 14035 were used. After overnight cultivation of both the strains the total mRNA extracted and converted to total cDNA.
Results: By Relative Real-Time PCR analysis the most cholera toxin production in classical and El Tor strains was at pH 8.5 and 8, respectively.
Conclusions: Therefore, We may conclude that use of acidic diet will help in reduction of cholera toxin production.

Keywords:Vibrio cholerae; Cholera Toxin; Enterotoxin

1. Background

Vibrio cholerae is a gram-negative bacterium responsible for seven pandemics in the world which most of the cases caused by the O_{139} and O_1 strains (1). Cholera toxin is produced by a lysogenic phage called *CTX*ϕ which is located on chromosome of *Vibrio cholerae* and contain significant antigen. Cholera toxin has important role in diarrhea which increases cAMP in the cells of the intestinal entrocytes and excrete considerable quantity of water and electrolytes from the body through the stool (2). In 2005, *Vibrio cholerae* infects people in several provinces of Iran, Pakistan and Afghanistan (3, 4). Production of Cholera toxin occurs within the small and large intestine of humans and which depends on various parameters like pH (2). Medical references showed that the most quantity of toxin produced in alkaline conditions but the exact amount is not clear (5). In 1987, Iwanaga et al. showed that the concentration of bicarbonate ions with increased cholera toxin has a direct effect (6).

2. Objectives

Based on this, our study, aimed to evaluate cholera toxin production in acidic, alkaline and neutral conditions.

3. Materials and Methods

In this study, two standard strains of *Vibrio cholerae* O_1 biovar El Tor N16961 & *Vibrio cholerae* O_1 biovar Classic ATCC 14035 were used. Both strains were cultured separately on the Luria Bertani agar. 10 microliters of saline containing *Vibrio cholerae* (classical or El Tor strain) inoculated to 100 cc AKI medium with different pH conditions and incubated at 37°C till the OD_{600} reached 0.4 which contained 1.5×10^8 cfu/mL bacterial cell (6).

3.1. Total mRNA Determination

Total mRNA for each strain was determined using an RNeasy Protect Bacteria Mini kit and RNasy ® mini kit (Qiagen, Germany), and then total mRNA was extracted from each sample.

3.2. Determination of total cDNA

By reverse transcription quantiTect ® kit (Qiagen, Germany), with random primer at 42°C for 10 min, extracted total mRNA was converted to total cDNA. Four Primers were designed: *recA-f* (5'-ATTGAAGGCGAAATGGGCGATAG-3'), *recA-r* (5'-TACACATACAGTTGGATTGCTTGAG-3'), *ctxAB-f* (5'-TATGCCAAGAGGACAGAGTGAG-3')

and *ctxAB-r* (5'-AACATATCCATCATCGTGCCTAAC-3') (7). *ctxAB* and *recA* gene-specific primers designed as the gene of interest and internal control, respectively.

3.3. Quantitative PCR

A SYBR Green qPCR assay was performed in a 20 μL containing 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany), 0.25 μM each specific primer and 4 μL of cDNA as a template. qPCR was performed as follows: one cycle of 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds , extension at 60°C for 30 seconds, and a final extension at 72°C for 2 minutes. Data acquisition, amplification of the primers, and relative expression analysis were carried out in a qPCR Corbett rotor gene-6000 detector.

3.4. Determination of Cholera Toxin Production

Following amplification, melting-curve analysis of the PCR products was carried out to

determine the specificity of the qPCR using genomic DNA for each gene to confirm that the primers amplified at the same rate and to validate the experiment. Relative expression levels of cholera toxin at various pH conditions were performed using the $2^{-\Delta\Delta C_T}$ method (8). In the qPCR, a negative control of distilled water was included in each run. Each sample was tested for the pentagram.

4. Results

The amplicons obtained for the *ctxAB* (115 bp) and *recA* (106 bp) genes were verified by sequencing. PCR yields were between 1.90 to 1.94, which was considered as the future of computing. Using analysis melting curve clearly indicates that only one band is formed in the qPCR process (Figure 1). The Cycle Threshold (CT) results are showing in Table 1. The C_T content for classical and El Tor strains were 20.79 ± 0.28 and 22.76 ± 0.12, respectively.

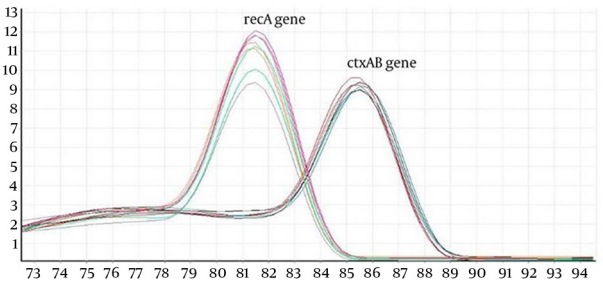

Figure 1. Melting Curves of *ctxAB* and *recA* genes

Table 1. Cycle Threshold (C_T) Results for the *Vibrio cholerae* O_1 Biovar El Tor N16961 & *Vibrio cholerae* O_1 biovar Classic ATCC 14035 [a]

Sample	pH	C_T (*recA*) ± SD	C_T (*ctxAB*) ± SD	Ratio	Sample	pH	C_T (*recA*) ± SD	C_T (*ctxAB*) ± SD	Ratio
***Vibriocholerae* O_1 Classic ATCC 14035, 1.5×10^8 cfu/mL as calibrator**	7	24.25 ± 0.10	24.39 ± 0.21	1	***Vibriocholerae* O_1 biovar El Tor N16961, 1.5×10^8 cfu/ml as calibrator**	7	24.50 ± 0.22	27.66 ± 0.32	1
Classic, 1.5×10^8 cfu/mL	5.5	NM	NM	NM	**El Tor, 1.5×10^8 cfu/mL**	5.5	NM	NM	NM
Classic, 1.5×10^8 cfu/mL	6	24.98 ± 0.23	31.56 ± 0.37	0.43	**El Tor, 1.5×10^8 cfu/mL**	6	24.94 ± 0.16	32.94 ± 0.15	0.38
Classic, 1.5×10^8 cfu/mL	6.5	25.11 ± 0.34	26.27 ± 0.22	0.75	**El Tor, 1.5×10^8 cfu/mL**	6.5	24.11 ± 0.33	29.87 ± 0.29	0.74
Classic, 1.5×10^8 cfu/mL	7	24.77 ± 011	24.39 ± 0.21	1	**El Tor, 1.5×10^8 cfu/mL**	7	24.66 ± 0.25	27.66 ± 0.32	1
Classic, 1.5×10^8 cfu/mL	7.5	24.56 ± 0.18	23.78 ± 0.23	1.05	**El Tor, 1.5×10^8 cfu/mL**	7.5	24.79 ± 0.19	24.89 ± 0.24	1.23
Classic, 1.5×10^8 cfu/mL	8	24.91 ± 0.27	22.29 ± 0.18	1.18	**El Tor, 1.5×10^8 cfu/mL**	8	25.18 ± 0.37	22.76 ± 0.12	1.45
Classic, 1.5×10^8 cfu/mL	8.5	23.97 ± 0.18	20.79 ± 0.28	1.32	**El Tor, 1.5×10^8 cfu/mL**	8.5	25.13 ± 0.17	23.12 ± 0.33	1.29
Classic, 1.5×10^8 cfu/mL	9	24.10 ± 0.20	23.74 ± 0.21	1.08	**El Tor, 1.5×10^8 cfu/ml**	9	25.07 ± 0.23	25.12 ± 0.27	1.11
Classic, 1.5×10^8 cfu/mL	9.5	24.77 ± 0.19	25.95 ± 0.29	0.95	**El Tor, 1.5×10^8 cfu/mL**	9.5	24.79 ± 0.11	28.12 ± 0.17	0.90
Classic (1.5×10^8 cfu/mL)	10	25.26 ± 0.37	30.09 ± 0.27	0.61	**El Tor, 1.5×10^8 cfu/mL**	10	24.87 ± 0.39	33.08 ± 0.17	0.71
Classic, 1.5×10^8 cfu/ml	10.5	24.12 ± 0.17	37.23 ± 0.19	0.12	**El Tor, 1.5×10^8 cfu/mL**	10.5	25.05 ± 0.29	38.77 ± 0.27	0.22
Classic, 1.5×10^8 cfu/mL	11	NM	NM	0	**El Tor, 1.5×10^8 cfu/mL**	11	NM	NM	0

[a] Abbreviations: NM, Not Measurable.

5. Discussion

As many scientists reported that cholera toxin is the main factor of *Vibrio cholerae* causing diarrhea which its production can be affected by various parameters (6). In the present study, evaluation of cholera toxin expression is carried out for the first time at different pH. As shown in Table 1 the maximum expression in El Tor and classical was 8.5 and 8, respectively. Our previous study in 2013 showed that, the expression of cholera toxin in El Tor strains is somewhat different from the classical strain (9). It seems that the expression of cholera toxin by classical strain in alkaline conditions is better than El Tor strain. This can confirm the Iwanaga et al. report regarding cholera toxin production is maximum by addition of bicarbonate in AKI medium. It is worth to note that bicarbonate can affect the toxT gene expression (6). As others reported that may factors may be involved in the expression of cholera toxin we for the first time determined that the cholera toxin production is maximum at 8-8.5 pH for both the strains. We suggest thus, according to the results it seems that, the consumption of acidic foods and probiotics can be effective in decreasing symptoms caused by *Vibrio cholerae*.

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