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Evaluation of Cholera Toxin Expression in Different Populations of *Vibrio cholera*

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Background: Cholera is one of the most diseases of human. Cholera toxin is the most important pathogenic factor in humans that causes diarrhea. The cholera toxin is produced by *V. cholerae* and CTXϕPhage.
Objectives: In this study, we have investigated the production cholera toxin with different density of *Vibrio cholerae*.
Materials and Methods: With this propose we inoculated classical strain O_1 of *Vibrio cholerae* ATCC 14035 and *Vibrio cholerae* O_1biovar El Tor N16961 into the AKI medium. Then, the total mRNA was determined by standard procedure which was converted into total cDNA.
Results: Cholra toxin production was determined by qPCR and maximum production of cholera toxin was at 10^{10} cfu/mL.
Conclusions: In conclusion, production of cholera toxin was minimized almost up to zero at $10^{10.5}$ cfu/mL; which could be due to presence of high level concentration of autoinducer.

Keywords:Vibrio cholerae; Cholera Toxin; Populations

1. Background

Cholera is a serious disease which is common all over the world particularly in developing countries (1). *Vibrio cholerae* is the causative agent of cholera; which can cause a considerable difficulties like diarrhea, dehydrates the body, acidosis, shock and ultimately death (2). The burden of this disease is well documented in the world and so far, seven global pandemic of cholera is reported (3, 4). As many scientists from all over the world believe that many pathogenic bacteria have the quorum sensing system which is one of the major virulence factors (5). In this system, as the number of bacterium increases, concentration of autoinducer will also increase; so, autoinducer concentration is directly related to the number of bacteria. Usually, among bacteria whenever, autoinducer concentration at its maximum level, quorum sensing process occurs; which may leads to the expression of bacterial virulence genes (6). However, this system in *Vibrio cholerae* is exactly the opposite (7). As Zhu et al. reported at low density of *Vibrio cholerae*, the autoinducer concentration is very low or not at all present; therefore, HapR protein is inactive. They believe, whenever, HapR protein is inactive and therefore, results in expression of ToxT protein which is responsible for cholera toxin expression (8).

2. Objectives

Based on above mentioned literatures, we aimed to study, cholera toxin production with different density of *Vibrio cholerae* population.

3. Materials and Methods

Vibrio cholerae O_1biovar El Tor N16961 and *Vibrio cholerae* O_1 Classic ATCC 14035 were donated by Iran institute Pasteur and maintained on conservative medium at -80°C. *Vibrio cholerae* O_1biovar El Tor N16961 and *Vibrio cholerae* O_1 Classic ATCC 14035 were inoculated to AKI medium for 24 hours during which every 10-15 minutes OD was measured and also cfu/mL was calculated.

3.1. Total mRNA Determination

The total mRNA was extracted by RNasy ® mini kits and Reagent RNAprotect ® bacteria kit (Qiagen, Germany) according to the manufacture's protocol.

3.2. Determination of total cDNA

The total cDNA was prepared from the total mRNA by using Reverse Transcription QuantiTect® (Qiagen, Germany) according to the manufacture's protocol.

3.3. Quantitative PCR

Primers used in this study are in Table 1. *recA* and *ctxAB* which is used in this study are as internal control and gene of interest respectively.

3.4. Determination of Cholera Toxin Production

Cholera toxin production was done according to QuantiTect SYBR Green PCR Master Mix Kit protocol (Qiagen, Germany), in quintuplet form.

4. Results

In this study, evaluation of cholera toxin gene expression was done by relative qPCR method. Based on gel electrophoresis our results lead to appearance of and *ctxAB* and amplified a 106 for *recA* and 115 bp for *ctxAB* genes respectively. All amplicons obtained for *recA* and *ctxAB* were verified by sequencing. For the above mentioned genes the efficiency was between 1.91-1.93. Melting curve analysis for *ctxAB* and *recA* was shown in Figure 1. In each strain, by using the $2^{-\Delta\Delta C_T}$ Method (9), maximum expression of cholera toxin was 0.90 with 10^{10}cfu/mL; and minimum expression of cholera toxin was 0.22-0.06 with $10^{10.5}$-10^{12}cfu/mL (Table 2).

Table 1. Sequences of Primers Used in This Study

Target Gene	Sequence 5' →3'	Reference
recA-f	ATTGAAGGCGAAATGGGCGATAG	(3)
recA-r	TACACATACAGTTGGATTGCTTGAG	(3)
ctxAB-f	TATGCCAAGAGGACAGAGTGAG	(3)
ctxAB-r	AACATATCCATCATCGTGCCTAAC	(3)

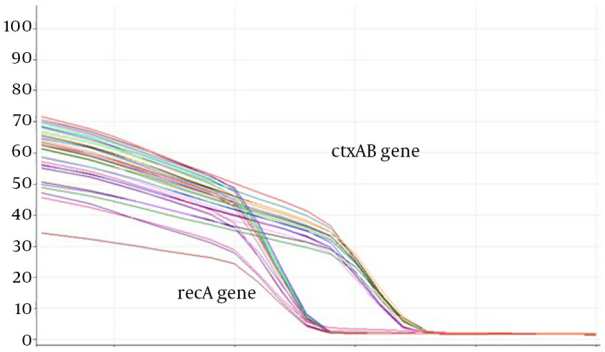

Figure 1. Melting Curves of *ctxAB* and *recA* Genes

Table 2. The qPCR Results and Its Analysis [a]

Sample	C_T (*recA*) ± SD	C_T (*ctxAB*) ± SD	Ratio	Sample	C_T (*recA*) ± SD	C_T (*ctxAB*) ± SD	Ratio
***Vibrio cholerae* O_1 Classic ATCC 14035, 1.5 × 10^8cfu/mL as calibrator**	24.25 ± 0.10	24.39 ± 0.21	1	***Vibrio cholerae* O_1 biovar El Tor N16961, 1.5 × 10^8cfu/mL as calibrator**	24.50 ± 0.22	27.66 ± 0.32	1
Classic, 10^2cfu/mL	NM	NM	NM	**El Tor, 10^2cfu/mL**	NM	NM	NM
Classic, 10^3 cfu/mL	NM	NM	NM	**El Tor, 10^3cfu/mL**	NM	NM	NM
Classic, 10^4 cfu/mL	32.56 ± 0.30	38.33 ± 0.22	0.92	**El Tor, 10^4cfu/mL**	33.75 ± 0.11	39.46 ± 0.11	0.96
Classic, 10^5cfu/mL	30.34 ± 0.23	36.11 ± 0.36	0.94	**El Tor, 10^5cfu/mL**	32.75 ± 0.33	38.12 ± 0.23	0.93
Classic, 10^6cfu/mL	28.77 ± 0.16	35.01 ± 0.35	0.94	**El Tor, 10^6 cfu/mL**	31.01 ± 0.27	37.77 ± 0.26	0.94
Classic, $10^{6.5}$cfu/mL	27.98 ± 0.32	33.88 ± 0.27	0.92	**El Tor, $10^{6.5}$cfu/mL**	29.54 ± 0.23	35.12 ± 0.12	0.88
Classic, 10^7cfu/mL	27.10 ± 0.31	31.20 ± 0.20	0.91	**El Tor, 10^7cfu/mL**	28.06 ± 0.19	33.44 ± 0.15	0.92
Classic, $10^{7.5}$cfu/mL	26.33 ± 0.12	29.78 ± 0.11	0.85	**El Tor, $10^{7.5}$cfu/mL**	27.65 ± 0.16	30.85 ± 0.27	0.90
Classic, 10^8cfu/mL	25.74 ± 0.25	26.99 ± 0.26	0.87	**El Tor, 10^8cfu/mL**	26.11 ± 0.22	27.93 ± 0.10	0.94
Classic, $10^{8.5}$cfu/mL	24.24 ± 0.18	25.10 ± 0.11	0.90	**El Tor, $10^{8.5}$cfu/mL**	25.18 ± 0.45	26.10 ± 0.20	0.83
Classic, 10^9cfu/mL	22.98 ± 0.17	22.96 ± 0.24	0.89	**El Tor, 10^9cfu/mL**	24.09 ± 0.18	25.11 ± 0.17	0.91
Classic, $10^{9.5}$cfu/mL	22.12 ± 0.18	22.12 ± 0.36	0.89	**El Tor, $10^{9.5}$cfu/mL**	23.12 ± 0.29	24.36 ± 0.13	0.89
Classic, 10^{10}cfu/mL	21.55 ± 0.19	20.88 ± 0.22	0.91	**El Tor, 10^{10}cfu/mL**	22.77 ± 0.25	23.17 ± 0.42	0.90
Classic, $10^{10.5}$cfu/mL	20.46 ± 0.42	29.11 ± 0.25	0.22	**El Tor, $10^{10.5}$cfu/mL**	21.96 ± 0.14	30.01 ± 0.19	0.24
Classic, 10^{11}cfu/mL	19.86 ± 0.22	34.33 ± 0.29	0.18	**El Tor, 10^{11}cfu/mL**	20.45 ± 0.26	34.56 ± 0.15	0.15
Classic, $10^{11.5}$cfu/mL	19.00 ± 0.13	37.96 ± 0.46	0.09	**El Tor, $10^{11.5}$cfu/mL**	20.12 ± 0.33	39.11 ± 0.31	0.08
Classic, 10^{12}cfu/mL	18.26 ± 0.14	39.56 ± 0.29	0.06	**El Tor, 10^{12}cfu/mL**	19.68 ± 0.28	41.23 ± 0.48	0.05

[a] Abbreviation: NM, Not Measurable.

5. Discussion

As we know, cholera toxin is a critical factor for diarrhea and cholera. Therefore, measurement of cholera toxin mRNA is equivalent to cholera toxin production (3). In 2002, Zhu et al. showed that, quorum sensing in *Vibrio cholerae* is opposite, exactly (8). Our results indicate that the suppression of cholera toxin production is in the range $10^{10.5}$cfu/mL for *Vibrio cholerae* O_1biovar El Tor N16961 and *Vibrio cholerae* O_1 Classic ATCC 14035. Therefore, as others also reported (10) that in this range, high level of autoinducer concentration suppressed the production of cholera toxin. This is also true in the case of El Tor strains. According to the result in Table 2, the production of cholera toxin in both classical and El Tor strains decreased gradually. This is due to reduced cholera toxin mRNA and decreased life span of cholera toxin. In conclusion, production of cholera toxin was minimized almost up to zero at $10^{10.5}$-10^{12}cfu/mL; which could be due to presence of high level concentration of autoinducer. We can suggest that genes involved in the production of autoinducer should be identified, cloned and expressed in *E. coli* in order to embed recombinant *E. coli* in the intestine of animals to evaluate their susceptibility to cholera.

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References

1. Taneja N, Mishra A, Sangar G, Singh G, Sharma M. Outbreaks caused by new variants of Vibrio cholerae O1 El Tor, India. *Emerg Infect Dis*. 2009;**15**(2):352–4.
2. Ritchie JM, Waldor MK. Vibrio cholerae interactions with the gastrointestinal tract: lessons from animal studies. *Curr Top Microbiol Immunol*. 2009;**337**:37–59.
3. Marashi SM, Bakhshi B, Fooladi AA, Tavakoli A, Sharifnia A, Pourshafie MR. Quantitative expression of cholera toxin mRNA in Vibrio cholerae isolates with different CTX cassette arrangements. *J Med Microbiol*. 2012;**61**(Pt 8):1071–3.
4. Bakhshi B, Pourshafie MR, Navabakbar F, Tavakoli A. Genomic organisation of the CTX element among toxigenic Vibrio cholerae isolates. *Clin Microbiol Infect*. 2008;**14**(6):562–8.
5. Lesic B, Starkey M, He J, Hazan R, Rahme LG. Quorum sensing differentially regulates Pseudomonas aeruginosa type VI secretion locus I and homologous loci II and III, which are required for pathogenesis. *Microbiology*. 2009;**155**(Pt 9):2845–55.
6. Willcox MD, Zhu H, Conibear TC, Hume EB, Givskov M, Kjelleberg S, et al. Role of quorum sensing by Pseudomonas aeruginosa in microbial keratitis and cystic fibrosis. *Microbiology*. 2008;**154**(Pt 8):2184–94.
7. Camara M, Hardman A, Williams P, Milton D. Quorum sensing in Vibrio cholerae. *Nat Genet*. 2002;**32**(2):217–8.
8. Zhu J, Miller MB, Vance RE, Dziejman M, Bassler BL, Mekalanos JJ. Quorum-sensing regulators control virulence gene expression in Vibrio cholerae. *Proc Natl Acad Sci U S A*. 2002;**99**(5):3129–34.
9. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;**25**(4):402–8.
10. Zhu L, Cai JP, Chen Q, Yu SY. Specific detection of toxigenic vibrio cholerae based on in situ PCR in combination with flow cytometry. *Biomed Environ Sci*. 2007;**20**(1):64–9.