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Original Article

Expression of α- and ε-toxin genes in *Clostridium perfringens* type D vaccine strain in contact with the Caco-2 cell line

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Abstract

Background: *Clostridium perfringens* commonly resides in the gastrointestinal tract and can survive in different environmental conditions. This pathogen produces several protein toxins including the potent ε-toxin which is classified as a category B toxin by the Centers for Disease Control and Prevention (CDC). In several studies, the induction of *C. perfringens* type C or D to produce toxins much more rapidly by close contact of bacteria with Caco-2 cells has been reported. **Aims:** The effect of close contact of enterocyte-like Caco-2 cells with *C. perfringens* type D (vaccine strain) on the production time of ε- and α-toxins was studied. **Methods:** During *C. perfringens* type D contact with Caco-2 cells for 5 h, ε- and α-toxins expressions (at 0, 2, and 5 h) were evaluated by a quantitative real-time PCR assay. Non-contacted bacteria with cells were included as the negative control in this research. **Results:** Bacterial contact with the Caco-2 cells induces a significant effect on the mean expression of the ε-toxin gene (*etx*) ($P<0.05$). Two h after contact, the highest level of gene expression was detected in the experimental group. Bacterial harvesting time, cell treatment, and their interactions did not affect significantly the mean expression of the *α*-toxin gene (*cpa*) ($P>0.05$). **Conclusion:** According to the findings of the present study, 2 h of bacterial contact with Caco-2 cells could stimulate *etx* gene expression in the *C. perfringens* type D vaccine strain.

Key words: Caco-2 cells, *Clostridium perfringens*, Gene expression, Toxin

Introduction

Clostridium perfringens is an anaerobic, Gram-positive, spore-forming bacillus, which commonly resides in the gastrointestinal tract, and can survive in different environmental conditions. This bacterium is widely spread around the world due to its high resistance to adverse environmental conditions, such as solar radiation, high atmospheric pressure, and low pH (Florence *et al*., 2011). *C. perfringens* is a well-known pathogen in livestock and humans, which can cause histotoxic and intestinal diseases, such as enteritis and enterotoxemia. Several reviews pointed out that most *C. perfringens* strains, including type A strains, produce numerous toxins and extracellular hydrolytic enzymes including 20 toxins and putative hydrolytic virulence factors (Uzal *et al*., 2014). Five toxin types of *C. perfringens* (A, B, C, D, and E) based on producing four major toxins (α-, β-, ε-, and ι-toxins) are outdated and based on a new scheme, seven toxin types of *C. perfringens* (A, B, C, D, E, F, and G) was introduced (Rood *et al*., 2018). The new toxin-based typing scheme of *C. perfringens* is shown in Table 1 (Revitt-Mills *et al*., 2015; Rood *et al*., 2018). Evidence suggests that CPA and CPB toxins can directly affect the cell membrane, leading to increased membrane permeability and disrupting the ionic balance in target cells. ITX toxin destroys the actin cytoskeleton of cells by activating intracellular reactions (Vidal *et al*., 2009). Besides, a high concentration of ETX toxicity increases the permeability of the intestinal mucous membrane; eventually, the toxin enters the bloodstream by passing through the mucous membrane. Moreover, ETX toxin leads to vascular edema in different tissues and causes necrotic and edema injuries by accumulating in tissues, especially in the kidneys and brain (Uzal *et al*., 2004). This toxin binds to specific membrane receptors on the surface of sensitive cells, forms a heptamer, and produces pores on the surface of cell membranes (Popoff, 2011). The membrane pores (diameter >2 nm) on the surface of mammalian cells facilitate the rapid passage of molecules with less diameter of 1 nm,

Table 1: *C. perfringens* toxin-based typing scheme [a]

Toxin type	α-toxin	β-toxin	ε-toxin	ι-toxin	CPE	NetB
A	+	–	–	–	–	–
B	+	+	+	–	–	–
C	+	+	–	–	+/–	–
D	+	–	+	–	+/–	–
E	+	–	–	+	+/–	–
F	+	–	–	–	+	–
G	+	–	–	–	–	+

[a] The names of structural toxin genes are shown in parentheses (Rood *et al*., 2018)

Table 2: The sequences and characteristics of primers used for the PCR of targeted genes in this study

Gene name	Primer sequence	Amplicon size	Reference
16S rRNA gene	F: 5-GGGGGTTTCAACACCTCC-3 R: 5-GCAAGGGATGTCAAGTGT-3	170	Matsuki *et al*. (2004)
Plc gene (α-toxin)	F: 5-AAGAACTAGTAGCTTACATATCAACTAGTGGTG-3 R: 5-TTTCCTGGGTTGTCCATTTCC-3	124	Schlegel *et al*. (2012)
etx gene (ε-toxin)	F: 5-TTTGATAAGGTTACTATAAATCCACAAGGA-3 R: 5-AGAGAGCTTTTCCAACATAAACATCTTC-3	121	Schlegel *et al*. (2012)

resulting in the decreased level of intracellular potassium ions and increased levels of intracellular sodium and chlorine ions, associated with membrane permeability disruption (Petit *et al*., 2003).

In some strains of this bacterium, sialidases have been shown to increase the binding and cytotoxic effects of ETX (Shimamoto *et al*., 2005). As contact is established between the bacterial strain and mammalian cells (e.g., enterocytes or Caco-2 cells), transcription and production of sialidase genes are increased. In some strains, trypsin increases the activity of some of these sialidase genes. Therefore, the presence of *C. perfringens* in proximity to intestinal trypsin triggers the production of sialidases, which in turn facilitates the binding of bacteria to enterocyte cells (Li *et al*., 2011).

C. perfringens uses a complex network of toxin gene expression regulators, including the two-component regulatory system (TCR) and accessory gene regulator (Agr)-like quorum sensing (ALQS), to regulate the gene expression of various toxins (Vidal *et al*., 2009; Ohtani, 2016; Ohtani and Shimizu, 2016). TCRs are two-component signal transmission systems, which consist of a membrane sensor and a cytoplasmic response regulator with environmental signals, such as light, temperature, food, and osmotic stress, which trigger the autophosphorylation of the membrane sensor (Chen *et al*., 2011).

The genome of *C. perfringens* encodes approximately 48 different TCRs (Shimizu *et al*., 2001). Most studies have been conducted on VirS/VirR systems, where VirS acts as a sensor histidine kinase, and VirR acts as a response regulator. After activating this system, VirR binds to special receptors on DNA, called VirR boxes, and contributes to the regulation of five different *C. perfringens* genes, including theta, α- (*cpa*), and β-2 (*β2*) toxin genes (Chen *et al*., 2011; Wang *et al*., 2012).

Additionally, the ALQS system is a cell density-dependent regulatory system. Many bacteria produce signals in the environment. When these signals reach the threshold level relative to the bacterial mass, bacteria produce an autoinducer. By the proper concentration of these molecules, bacteria respond to these signals by activating different signaling pathways, altering gene expression, and modulating the physiological processes (Chen *et al*., 2014).

A study on the ALQS system's role in regulating the expression of important toxins indicated that this system could be used appropriately for therapeutic purposes (Gray *et al*., 2013). In this regard, Chen *et al*. (2011) showed an increase in ETX production due to the contact of *C. perfringens* type D and B with the Caco-2 cell line. In the Razi Vaccine and Serum Research Institute (RVSRI) of Iran, toxins are the main components of enterotoxemia vaccines; therefore, the improvement of toxin production capacity can enhance the production of enterotoxemia vaccines. The present study aimed to investigate the effect of *C. perfringens* vaccine strain exposure to the Caco-2 cells on the expression of *cpa* and *etx* genes, as well as the maximum time of increased toxin expression after exposure.

Materials and Methods

Primary genomic evaluation of studied strain

Initially, provided standard strain of *C. perfringens* type D by Ahvaz Branch of RVSRI, was evaluated for the presence of α- and ε-toxin genes and absence of β-toxin gene by conventional PCR. This stage was done for two reasons: First, confirmation of the presence of two studied genes (α- and ε-toxin genes), and second, partially toxin typing of the studied strain. Data of the used oligonucleotide primers are demonstrated in Table 2. The common PCR thermal cycle program of α- and ε-toxin genes was as follows: initial denaturation at 95°C for 10 min, 30 cycles containing denaturation at 94°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s, followed by a final extension at 72°C for 5 min.

The PCR thermal cycle program of the β-toxin gene was as follows: initial denaturation at 95°C for 10 min, 35 cycles containing denaturation at 95°C for 60 s, annealing at 61°C for 60 s, and extension of 72°C for 60 s, followed by a final extension at 72°C for 10 min. Both PCRs were performed in a final volume of 20 µL, consisting of 10 µL of Master Mix (Ampliqon), 1 µL of each primer (10 µM), 2 µL of DNA, and 6 µL of distilled water. Finally, the PCR products were electrophoresed using 1% agarose gel, containing 3 µL/100 ml Safe Stain (Sinaclon, Iran).

Preparation of Caco-2 cell line

The Caco-2 cell line was provided by the National Center for Genetic and Biological Reserves of Iran. Cells were cultured in RPMI-1640 medium, containing 10% fetal bovine serum (FBS), ciprofloxacin (10 µg/ml), piperacillin (10 µg/ml), and gentamicin (10 µg/ml), and incubated at 37°C in an atmosphere of 5% CO_2. After 48 h, the cells were passaged, using an EDTA-trypsin solution. To improve the enzyme function, incubation was performed at 37°C for 5 min.

After ensuring the detachment of cells using an inverted microscope (Olympus, Japan), a culture medium containing FBS was added to the flask. Next, using a Neobar lam, the cells were counted, and a cell dilution (7×10^5 cells/ml) was prepared. Then, the cells were cultured in 24-well plates. The culture medium was replaced every day, and the cells were examined under an inverted microscope (Olympus, Japan) until reaching 100% confluency.

Preparation of *C. perfringens* type D

First, a lyophilized vial containing *C. perfringens* type D vaccine strain (RVSRI, Iran) was inoculated under anaerobic conditions provided by Anoxomat System (MART® Microbiology BV, Netherlands), in a fresh calf liver extract medium for 24 h at 37°C. Next, a certain amount of the bacterial culture medium was transferred to a thioglycolate broth medium and stored at 37°C for 24 h under anaerobic conditions. Then, the bacterial culture was centrifuged at 8000 ×g for 20 min at 4°C, and the bacterial deposit was rinsed twice with a cold phosphate-buffered saline (PBS) solution (pH=7.4). Finally, the bacteria were suspended in the PBS solution, containing 0.1% cysteine (1.5×10^7 CFU/ml), and immediately transferred to the cell culture wells, containing Caco-2 cells for simulating an infection.

Contact of *C. perfringens* with Caco-2 cell culture

To investigate the effect of contact duration on the gene expression of the studied toxins, sampling was performed at 0, 2, and 5 h post-contact. To reduce errors, the experiments were conducted in triplicate. Six wells were considered in each sampling time (i.e., 0, 2, and 5 h), the first to third wells containing 1 ml of *C. perfringens* (1.5×10^7 CFU/well) in contact with Caco-2 cells. The culture medium of the cells did not contain any antibiotics for this group. The fourth to sixth wells containing 1 ml of *C. perfringens* at a predetermined dilution without Caco-2 cells considered as the control group.

For sampling of all experimental groups, the supernatant of each well was collected in a sterile microtube. Samples were centrifuged at 5000 rpm for 8 min. The sedimented bacteria were washed using 1 ml of PBS, containing 0.1% cysteine, and stored at -70°C until the time of real-time polymerase chain reaction (RT-qPCR) assay.

To demonstrate bacteria and Caco-2 cell contact, some wells were stained with Giemsa. These wells were fixed with absolute methanol. After 15 min, methanol was discharged, and the wells dried at room temperature. Then, fixed cells were stained with 1/20 diluted commercial Giemsa solution for 10 min, washed with distilled water, and observed under an inverted microscope (Nikon, USA). The wells containing Caco-2 cells with and without bacteria were compared.

RNA extraction and cDNA synthesis

In this study, RNA was extracted from bacterial cells using a commercial RNA extraction solution (RNX-Plus, CinnaGen, Iran) according to the manufacturer's instructions. Before cDNA synthesis, the quality and quantity of the extracted RNA were evaluated by 1% agarose gel electrophoresis, and NanoDrop spectrophotometry (Thermo Scientific, USA) at a wavelength of 260 nm. To measure the amount of extracted RNA and its purity, 1 µL of each RNA was evaluated in a NanoDrop system, and its purity was evaluated by measuring the ratio of absorbance at 260 and 280 nm (A260/280). For all extracted RNAs, the A260/280 ratio was between 1.8 and 2.1.

The first cDNA strand was synthesized using a cDNA synthesis kit (Sinaclon Co., Iran) containing random hexamer primers. To synthesize 20 µL cDNA of each sample, 5 µL of RNA at a concentration of 0.5 µg and 1 µL of random hexamer primer were added to a microtube, and the volume was brought up to 12 µL using nuclease-free water. The microtube was placed at 65°C for 5 min and cooled at 37°C. Then, 4 µL of reaction buffer, 2 µL of dNTP at a concentration of 10 µmol, and 1 µL of Ribolock™ RNase Inhibitor were added to the microtube. After 5 min incubation at room temperature, 1 µL of M-MuLV reverse transcriptase enzyme was added and mixed. The final reaction volume was 20 µL.

In the next step, the microtube was placed at 42°C for 1 h to synthesize cDNA, followed by 70°C incubation for 10 min to inactivate the enzyme.

Conventional PCRs for *cpa*, *etx*, and *16S rRNA* genes

To evaluate the accuracy of cDNA and primer synthesis, conventional PCRs for *cpa*, *etx*, and *16S rDNA* genes were performed in a final volume of 25 µL consisting of 12.5 µL of Master Mix (Ampliqon), 1 µL of each primer (10 µM), 2 µL of cDNA, and 8.5 µL of distilled water. A common thermal cycle program for the

PCR assays was applied as follows: initial denaturation at 95°C for 10 min, 30 cycles including denaturation at 94°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s, followed by a final extension at 72°C for 10 min. Finally, the PCR products were evaluated using 1% agarose gel, containing 3 µL/100 ml Safe Stain (Sinaclon, Iran). The sequences and characteristics of primers used for *cpa*, *etx*, and *16S rRNA* genes are presented in Table 2.

Evaluation of gene expression by RT-qPCR assay

To evaluate the expression of *cpa* and *etx* genes in the contact and control groups, an RT-qPCR assay was performed using the SYBR® Green method in a StepOnePlus Real-Time PCR System (Applied Biosystems, USA). In this study, a standard plot was depicted for each gene to investigate the efficiency and other characteristics of RT-qPCR reactions. For relative quantification, the housekeeping gene of *16S rRNA* was used as a reference gene to normalize RT-qPCR data.

To perform the reaction, 12.5 µL of Master Mix (Ampliqon), 1 µL of each primer, and 2 µL of cDNA were added to each microtube, and the volume was brought up to 25 µL using nuclease-free distilled water. RT-qPCR was performed in triplicate, according to the protocol described earlier. The melting curve was also plotted to investigate the denaturation temperature of real-time PCR products. Control group was indicated as the calibrator in the analysis of data. The expression ratio between each test sample and the calibrator one was estimated using the $2^{-\Delta\Delta CT}$ formula, based on the method proposed by Pfaffl *et al*. (2002).

Statistical model and data analysis

The experiments were carried out in a completely randomized design with a 3 × 2 factorial arrangement (6 treatments and 3 replications). The experimental factors included two differently treated bacteria (contact with Caco-2 and non-contact with Caco-2) and 3 different harvesting times (0, 2, and 5 h post-exposure). The RT-qPCR results were analyzed in SAS ver. 9.1 (2004) using the general linear model (GLM) procedure. Besides, Duncan's multi-range test was used for mean comparisons. A P-value less than 0.05 was considered statistically significant.

Results

Based on the primary genomic evaluation of the studied strain by PCR, the presence of *α*- and *ε*-toxin genes and the absence of *β*-toxin gene were confirmed (Fig. 1). Giemsa staining of cells for observation of connection between Caco-2 cells and bacteria was demonstrated in Fig. 2.

Figure 3 represents the gel electrophoresis results of extracted RNA in the groups of exposed and nonexposed bacteria to Caco-2 cells, and no degraded RNA was detected in the gel. NanoDrop of extracted RNAs showed that all sample's A260/280 ratios were between 1.8 and 2.1.

The PCR results of *cpa*, *etx*, and *16S rRNA* genes were shown in Fig. 4. Single bands with the sizes of 124 bp, 121 bp, and 170 bp are observed in the gel picture related to the *cpa*, *etx*, and *16S rRNA* genes, respectively.

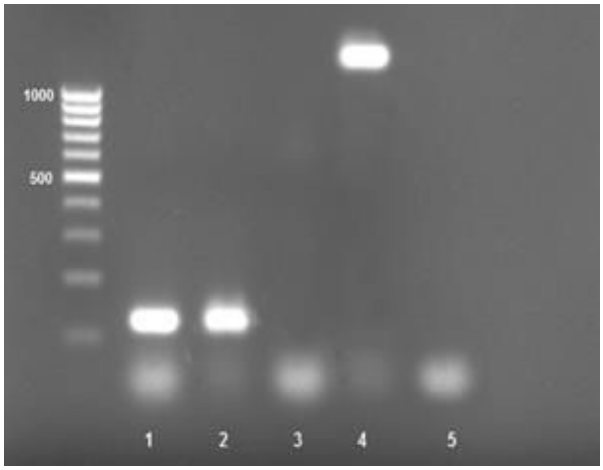

Fig. 1: Electrophoresis of PCR products of *C. perfringens* type D. First Lane: Ladder 100 bp, Lane 1: *α*-toxin gene (124 bp), Lane 2: *ε*-toxin gene (121 bp), Lane 3: *β*-toxin gene, Lane 4: *β*-toxin gene, as positive control (1421 bp), and Lane 5: No template control (NTC)

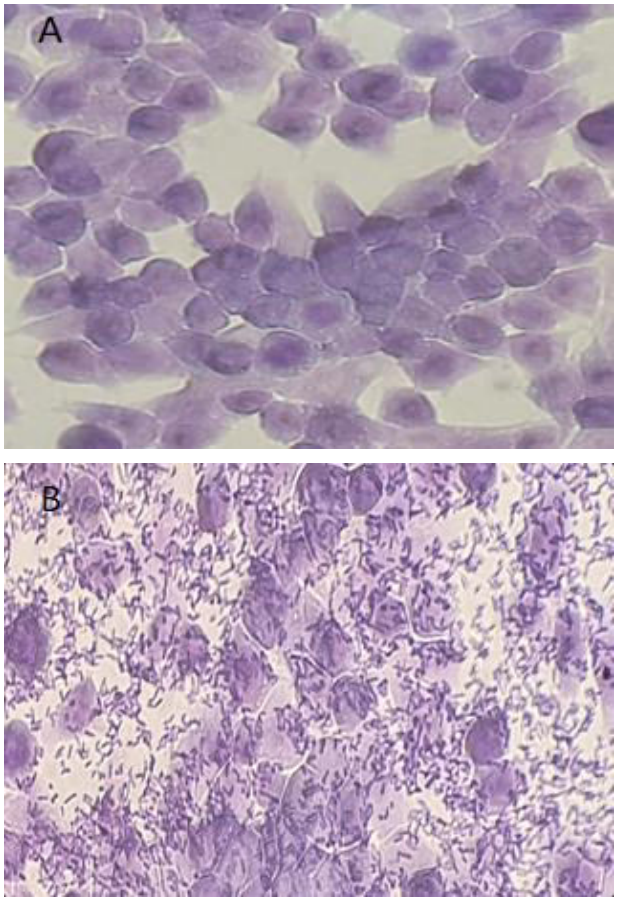

Fig. 2: Microscopic images of contacted and non-contacted cells. Giemsa staining of non-contacted (**A**), and contacted (**B**) Caco-2 cells with *C. perfringens* type D

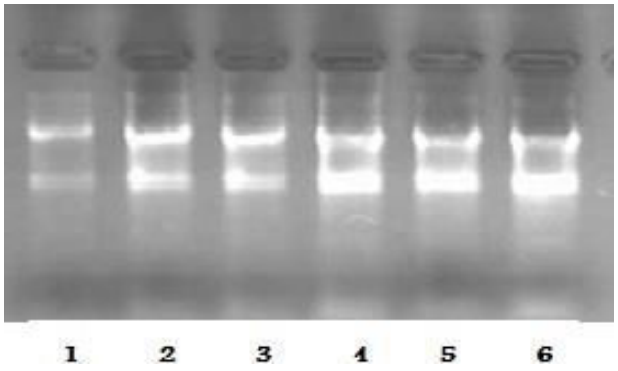

Fig. 3: Samples of electrophoresis of total RNA extracted from *C. perfringens* type D on a 1% agarose gel. Lanes 1-3: Bacteria in contact with Caco-2 cells, and Lanes 4-6: Bacteria not in contact with Caco-2 cells

Melting curves of RT-qPCR reactions are shown in Fig. 5. Also, the presence of 3 distinct peaks in the melting curve of *cpa*, *etx*, and *16S rRNA* genes confirms the specificity of primers for these 3 genes (Fig. 5).

The effect of exposure duration (0, 2, and 5 h) on the *etx* gene expression is presented in Table 3. The duration of bacterial contact with the Caco-2 cells induced a significant effect on the mean expression of *etx* gene (P<0.01). The mean expression of *etx* gene in the experimental group harvested at 2 h, and 5 h after exposure was significantly different from the mean expression level at 0 h. The highest level of gene expression was observed in the experimental group at 2 h; however, the difference between the intervals of 2 h and 5 h was not statistically significant (Table 3).

Table 3: The analysis of time treatment effects on the expression of ε-toxin gene (P<0.05)

Duncan grouping *	Mean±SEM	Harvest time (h)
B	2.712 ± 0.11	0
A	3.075 ± 0.11	2
A	3.333 ± 0.11	5

* The means with the same letter are not significantly different and the means with dissimilar letters are significantly different. SEM: Standard error mean

The effect of *C. perfringens* type D in contact with the Caco-2 cells on the *etx* gene expression is presented in Table 4. The effect of exposure to Caco-2 cells on the *etx* gene expression was statistically significant (P<0.01). The highest level of gene expression was observed in the experimental group of contact with the cells.

Table 4: The analysis of cell treatment effects on the expression of ε-toxin gene (P<0.05)

Duncan grouping *	Mean±SEM	Cell treatment
B	2.669 ± 0.09	No cells
A	3.411 ± 0.09	Cells

* Means with dissimilar letters are significantly different. SEM: Standard error mean, Cells: Representing *C. perfringens* type D contact with Caco-2 cells, and No cells: Representing *C. perfringens* type D not in contact with Caco-2 cells

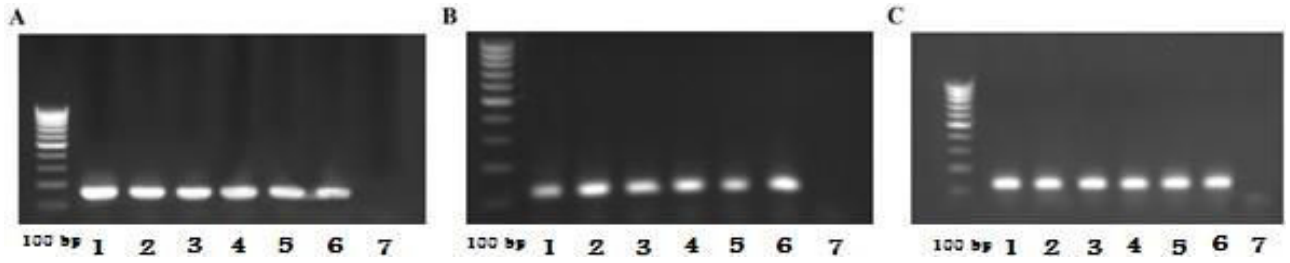

Fig. 4: Electrophoresis of PCR products of the cDNA sample of *C. perfringens* type D. (**A**) *16S rRNA* gene, (**B**) α-toxin gene, and (**C**) ε-toxin gene on 1% Agarose gels. Lanes 1-3: Bacteria without contact with Caco-2 cells at 0, 2, and 5 h, respectively, Lanes 4-6: Bacteria in contact with Caco-2 cells at 0, 2, and 5 h, respectively, and Lane 7: Negative control

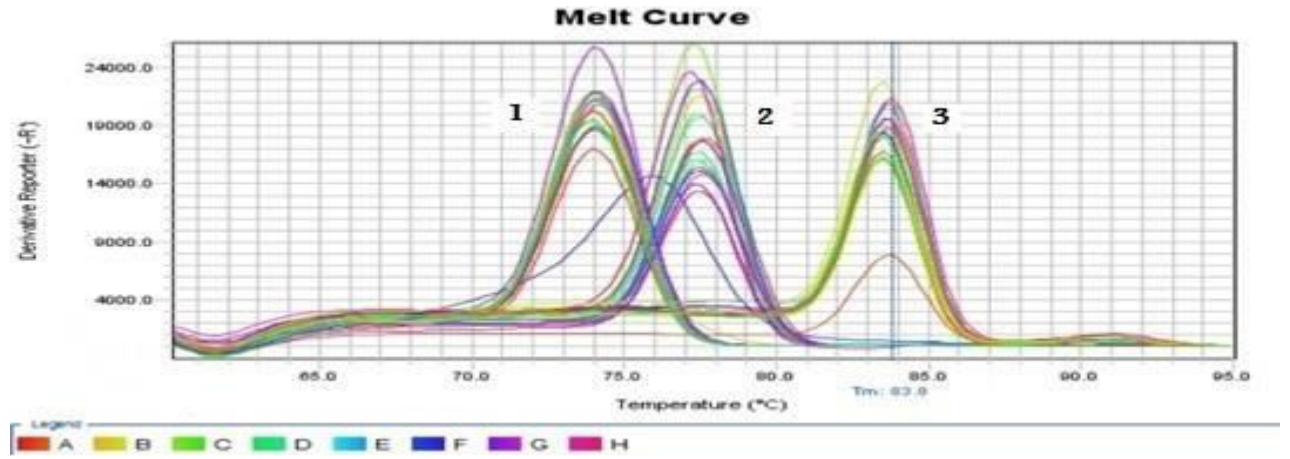

Fig. 5: Melting curve analysis of SYBR Green real-time PCR products. The melting curves for α-toxin (**1**), ε-toxin (**2**), and *16S rRNA* genes (**3**)

Table 5: Interaction of cell treatments and time on ε-toxin gene expression (P<0.05)

Time (h)	0		2		5		SEM	P-value
Bacterial treatments	Contact with Caco-2	Non-contact with caco-2	Contact with Caco-2	Non-contact with caco-2	Contact with Caco-2	Non-contact with caco-2		
Mean gene expression	2.720[a]	2.703[a]	3.623[b]	2.527[a]	3.890[b]	2.777[a]	0.15	<0.001

The means with dissimilar letters have significant differences based on Duncan's multiple range tests and the means with the same letter are not significantly different. SEM: Standard error mean

Table 6: Interaction of cell treatments and time on α-toxin gene expression (P<0.05)

Time (h)	0		2		5		SEM	P-value
Bacterial treatments	Contact with Caco-2	Non-contact with caco-2	Contact with Caco-2	Non-contact with caco-2	Contact with Caco-2	Non-contact with caco-2		
Mean gene expression	5.100[b]	4.140[a]	4.347	4.153	4.303	4.790	0.28	0.072

The means with dissimilar letters have significant differences based on Duncan's multiple range tests and the means with the same letter are not significantly different. SEM: Standard error of the mean

Moreover, the effect of interaction duration between *C. perfringens* type D bacteria and the Caco-2 cells on *etx* gene expression is presented in Table 5. The mean expression of *etx* gene showed significant differences in all experimental groups (P<0.05). The expression level of *etx* gene in the contact groups (with Coco-2 cells) at 2 h and 5 h significantly increased compared to those at 0 h and compared to the control group (without contact). An increase in the *etx* gene expression was observed in the contact groups at 0, 2, and 5 h. However, the increase in the *etx* expression of the contact group at 5 h was not statistically significant compared to those at 2 h post-exposure (Table 5).

Additionally, the expression level of the *C. perfringens cpa* gene was measured using the RT-qPCR. The results showed that the effects of bacterial harvesting time, cell treatment, and their interactions were not statistically significant on the mean expression of *cpa* gene (P<0.05); however, the effects of contact time and cell treatment are not shown here. The effect of interaction duration between *C. perfringens* type D bacteria and Caco-2 cells on the mean expression of *cpa* gene is shown in Table 6. The difference between the contact group and without cell group was only significant at 0 h.

Discussion

As the pathogenesis of bacteria relies on toxin production, studies on the expression regulation of clostridial toxin genes in the presence of host cells and on the evaluation of toxin production by *Clostridium* spp. *in vivo* are very important. Enterotoxemia is endemic in Iran, and millions of vaccine doses are annually produced by the RVSRI in Iran. However, given the vaccine toxicity, identification of factors that increase the expression of toxin genes (ε and α) effectively, and determination of their expression peak time can be effective in the production of more cost-effective vaccines.

According to *in vitro* studies, the maximum production of *C. perfringens* type C toxin mainly occurs in the late logarithmic phase (Vidal *et al*., 2009). This finding confirmed the lack of toxin production in the early hours of the bacterium type C cultured on the trypticase-glucose-yeast extract (TGY) medium (Fisher *et al*., 2006). On the other hand, Vidal *et al*. (2009) showed that the presence of intestinal Caco-2 cells leads to the rapid expression of *C. perfringens* type C toxin genes. The rapid pathogenesis of *C. perfringens* type C in the intestines of humans and animals, as necrotic enteritis, leads to the patient's death within 48 h. During intestinal infection, faster production of toxins leads to faster absorption of toxins and ultimately results in the faster manifestation of symptoms (Amimoto *et al*., 2007).

Studies on *C. perfringens* type C and D in contact with Caco-2 cells showed that their interaction requires close, but not solid, contact (Vidal *et al*., 2009). It has been also reported that this contact does not stimulate the growth of *C. perfringens* (Vidal *et al*., 2009). The results of this study showed a significant increase in the *etx* gene expression, but the expression of *cpa* gene was not significant within 5 h (P<0.01). In other words, there was no increase in the expression of *cpa* gene in any of the studied intervals. The contact of type D cells with Coca-2 cells did not stimulate an increase in *cpa* gene expression.

Several studies have investigated the expression of different toxin genes (ε, α, and enterotoxin) in various types of *C. perfringens* (Gkiourtzidis *et al*., 2001). In this regard, Chen *et al*. (2011) reported that contact of *C. perfringens* type D strain CN3718 with enterocyte-like Caco-2 cells led to the regulation of ε-toxin production. By studying the expression of *etx* gene in Type D of CN3718 strain, they found that the expression of this toxin was associated with the expression of *agr* operon and the QS phenomenon. However, the production of this toxin was not affected by the VirS/VirR regulatory system in this strain.

Moreover, Vidal *et al*. (2009) reported the effect of enterocyte-like Caco-2 cells on the positive regulation of *cpa* gene in *C. perfringens* type C strain CN3685. An increase in the expression of this toxin gene was observed not only by Caco-2 cells but also by 1R-12 fibroblast cells in rats and MDCK cells in dog kidneys.

According to these results, the expression of toxin genes may also increase in non-intestinal infections, such as gas gangrene, which is caused by CPA and PFO toxicity (Lyristis *et al*., 1994). By investigating the expression of different genes of this bacterium, Vidal *et al*. (2009) reported an increase in *cpb*, *cpb*2, *plc*, and *pfo*A gene transcription. The current study revealed that stimulation of the expression of all type C toxins by the Caco-2 cells did not occur for 2 h. Similar results were reported for ε-toxin production by two isolates of *C. perfringens* type D and β-toxin production by *C. perfringens* type C, which did not indicate an increase in the expression of toxins by using Caco-2 cells at any of the hours after treatment (Vidal *et al*., 2009).

According to the findings of the present study, the exposure of Caco-2 cells with *C. perfringens* could reduce the stimulation time of *etx* gene expression to 2 h in the *C. perfringens* type D vaccine strain. Other factors, such as environmental factors affecting gene expression, and control systems of gene expression might contribute to this finding (Ohtani and Shimizu, 2016). The results of this study could be applied to the understanding of the pathogenesis of this bacterium, and making effective changes in the vaccine production process. The scarcity of information on the regulatory mechanism of the expression of each toxin in different types of *C. perfringens*, especially types B and D, indicates the need for further studies.

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Conflict of interest

The authors declare no conflict of interest.

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