Modulatory effect of *Vibrio cholerae* toxin co-regulated pilus on mucins, toll-like receptors and NOD genes expression in co-culture model of Caco-2 and Peripheral Blood Mononuclear Cells (PBMC)

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Abstract

Background: *Vibrio cholerae*, the causative agent of cholera, as a Gram-negative pathogen tend to colonize the small intestine. The intestinal mucus layer forms mucin physical barrier, consisted from high molecular weight proteins. Regarding the role of toxin–coregulated pilus (TCP) as one of the most important colonization factors of *V. cholerae*, this experimental study was designed to determine the role of TcpA in induction of mucin production and its regulatory effect on innate immunity molecules including TLRs and NODs using Caco2- PBMC cocultures as an interactive model.

Materials and methods: The rTcp protein was expressed in pET-28a-*tcpA* construct and purified using Ni-column chromatography. The identity of rTcp was confirmed by western immunoblotting analysis using anti-poly-histidine antibody. Nontoxic doses of rTcpA was determined on Caco-2 cell lines. The effects of different concentrations of rTcpA (1, 5, 10 and 50 µg/mL) on the expression of *mucin* 1,3, 4, toll-like receptors (TLR1, 4), and Nucleotide-binding oligomerization domain-containing proteins (NOD1, 2) genes were evaluated in a co-culture model of human colon carcinoma cell line (Caco-2) and Peripheral Blood Mononuclear cells (PBMCs).

Results: The rTcpA protein of *V. cholerae* was expressed in BL21 E. coli and confirmed by western blotting. The rTcpA showed a statistically significant effect on the expression of *muc* genes (MUC3 and MUC4) in a dose-dependent manner. This finding is supposed to facilitate physical adhesion and colonization of *V. cholerae* in intestinal lumen. The rTcpA moderately stimulated the expression of *tlr4* and overexpressed *tlr1*, both of which are supposed to induce a mucosal protective response against bacterial infection and would help a promising protection in prophylaxis applications. No change in NOD2 expression might be attributed to the non-invasive nature of *V. cholerae* as an intestinal pathogen.

Conclusion: In conclusion, the rTcpA protein of *V. cholerae* showed a statistically significant modulatory effect on the human gut epithelium gene expression which would help promising protection in prophylaxis applications.

Background

Cholera is an infectious disease which is caused by *Vibrio cholerae*, a Gram-negative rod in the family Vibrionaceae. *V. cholerae* strains responsible for cholera belong to O1 and O139 serogroups and tend to live in warm waters which can be transmitted to human due to poor sanitation [1]. This enteric pathogen has to move toward enterocytes and colonizes the small intestine. Consequently, the bacterium must overcome the intestinal physical barrier, including the mucus layer which covers the gastrointestinal tract [2]. The mucus layer mainly includes high molecular weight glycoproteins named mucins [3]. Oligosaccharide side chains of mucins entrap bacteria and immobilize them which preclude bacterial access to the epithelium and subsequent enterocyte damage [4]. Conversely, mucins can mediate an interaction between bacteria and epithelium which leads to intestinal colonization [5]. Mucins are classified into two subgroups: i) secreted gel-forming and ii) membrane-bound [6]. The enterocytes

represent membrane-bound mucins which make a glycocalyx on their apical surface and consist of the mucins 1,3,4,12,13 and 17 [7]. The MUC 3 and MUC 4 mucins are considered as two major components of glycocalyx [3]. The MUC 1 which is broadly distributed among different organs and represents a high level of expression by mucosal tissues [7, 8].

Even though cholera is known as a non-inflammatory disease but low-grade inflammation can be seen in the early stage of disease [9]. *V. cholerae* toxin induces a moderate inflammatory response in intestinal milieu mediated by Pattern Recognition Receptors (PRRs). The PRRs like toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLR) play a crucial role in microbial identification [10]. Epithelial cells also play an important role in defense against pathogenic bacteria by expression of TLR1,2,4,5 and TLR6 [11]. NOD-like receptors are another class of PRRs that are expressed by human cells. The important members of the NLRs family are NOD1 and NOD2 which have been known in macrophages, dendritic, Paneth, and intestinal epithelial cells. NLRs are contributed in the activation of caspase 1 and mediates pro-inflammatory cytokines IL-1 β and IL-18 secretion [12]. Likewise, innate immune responses are induced with TLRs [13].

V. cholerae TCP which mediates the attachment of *V. cholerae* to enterocytes and M cells, belongs to type IV pili [14]. TcpA is the main component of toxin co-regulated pilus and has approved a crucial role in *V. cholerae* colonization [15]. Regarding the pivotal role of TcpA in bacterial colonization, recognition of its potential role in mucin gene expression is compulsory.

This study aimed to determine the effect of rTcpA on the expression of mucin 1,3, 4, TLRs1, 4 and NOD 1, 2 genes in the interaction model of co-cultured human epithelial colorectal adenocarcinoma cells (Caco-2) and peripheral blood mononuclear cells (PBMC) which were treated with various concentration of recombinant TcpA.

Results

Cloning, expression and purification of rTcpA

The *tcpA* gene was amplified by PCR and the 650 bp product was sequenced and confirmed by NCBI (<http://www.ncbi.nlm.nih.gov/>). The *tcpA*-pET28a construct was transformed into *E. coli* BL-21, rTcpA was expressed and purified. The purified protein was viewed on SDS-PAGE with a size about 22 kDa and its identity confirmed by western blotting (Figure 1).

Cytotoxicity effect of rTcpA on Caco-2 cells

The cytotoxic effect of rTcpA was evaluated on Caco-2 cells by MTT assay. The rTcpA concentration of 0.1 μ g/mL inhibited 3% of Caco-2 cells in 24, 48 and 72 h. Whereas, the inhibitory effect was increased to 5%, 8% and 14%, when the Caco-2 cells were exposed to 1 μ g/mL of rTcpA for 24, 48 and 72 h, respectively. The cytotoxicity effect was determined as 8%, 18%, and 30%, for 24, 48 and 72 h exposure time, respectively, when increasing the rTcpA concentration up to 10 μ g/mL.

The effect of rTcpA on *mucin* genes expression in Caco-2/PBMCs co-culture by real-time PCR

The expression of *mucin1,3* and *4* genes was assessed in Caco-2/PBMCs co-culture by real-time PCR after treatment with different concentrations of rTcpA. The results demonstrated that the expression of *mucin 1* was significantly ($P<0.05$) increased to 2.3, 5.5, 7.5 and 8.6 folds in the presence of rTcpA1, 5, 10 and 50 μ g/mL, respectively compared to the untreated Caco-2/PBMC co-culture as control. Our data revealed a highly significant increase ($P<0.05$) in the expression level of *muc4* gene (3.8, 9.5, 14.9 and 185 folds) in the co-culture group which was exposed to 1, 5, 10 and 50 μ g/mL of rTcpA, respectively. With the same concentrations of rTcpA after 24 h exposure, 1.3, 7.6, 12.2 and 76.8 fold changes ($P<0.05$) was observed in *muc3* gene expression compared to untreated Caco-2/PBMC co-culture control group. The expression of all *muc* genes was statistically increased after treatment with different concentrations of rTcpA (Table 1).

The TLR1 and TLR4 genes expression of Caco-2/PBMCs co-culture in response to rTcpA

The expression level of *tlr* genes (*tlr1* and *4*) were evaluated in the co-culture model of Caco-2/PBMCs by real-time PCR when treated with different concentrations of rTcpA, considering untreated cells as control. The expression of *tlr1* was increased to 1.01, 1.93, 3.94 and 30.73 folds after exposure to 1, 5, 10 and 50 μ g/mL of rTcpA, respectively which showed a significant increase ($p<0.05$). The 0.309, 0.564, 1.16- and 4.962-folds changes were observed in *tlr4* gene expression when after 24 h treatment with 1, 5, 10 and 50 μ g/mL rTcpA, respectively ($p<0.05$) (Table 1).

The effect of rTcpA on NOD genes expression in Caco-2/PBMCs co-culture by real-time PCR

In this study, the expression of *nod1* was changed 0.177, 0.303, 0.33 and 0.37 folds in the Caco-2/PBMC co-culture medium after exposure to different concentrations of rTcpA (1, 5, 10 and 50 μ g/mL, respectively) which did not show a statistical significant difference ($P>0.05$). The expression of *nod2* gene was increased to 0.555, 2.35, 4.36 and 19.904 folds when treated by rTcpA 1, 5, 10 and 50 μ g/mL, respectively. The differences in *nod2* gene expression after exposure with different concentrations of rTcpA were statistically significant ($p<0.05$) (Table 1).

Discussion

The effect of TCP as an important virulence factor of *V.cholerae* on the expression of *muc*, *tlr* and *nod* genes was considered in this study. The MUC4 mucin, as the membrane-bound subgroup of mucins, play a critical role in enterocyte protection and indirect immune responses against infections [16, 17]. Our results showed that the expression of this protein was strongly stimulated by rTcpA in a co-culture model. It seems that TcpA protein probably facilitates physical adhesion of *V. cholerae* by augmentation of MUC4 mucin production by inducing intestinal enterocytes, which is supposed to facilitate bacterial colonization. Vieira et al.(2010) demonstrated that atypical Enteropathogenic *E. coli* strains (aEPEC) caused up-regulation of *muc4* expression and increased its

secretion followed by increased attachment and colonization of bacteria at the apical surface of HT29-MTX cells [18].

The expression of *muc3* gene was induced by rTcpA in the present study. It seems that this increase is also important in the adhesion stage of *V. cholerae* to Caco-2 cells. Bhowmick (2008) established that the chitin binding protein (*GbpA*) enhances both *muc3* expression and mucin secretion that leads to the initial colonization of *V. cholerae* [5]. Therefore, it can be concluded that these two binding factors, synergistically, empower bacteria for adherence to intestinal cells.

The critical role of MUC1 as a receptor for *Salmonella* adhesion (SiiE) has been confirmed in apical invasion of *Salmonella* into enterocytes [19]. On the other hand, it was demonstrated that overexpression of *mucin1* gene occurs in response to invasive intestinal bacterial infections, including *Campylobacter jejuni* which plays a serious role in limiting their pathogenesis [20]. The expression level of *muc1* was up-regulated by rTcpA, but it was affected less than MUC3 and MUC4. Considering the toxin producing and extracellular lifestyle of *V. cholerae* infection, the limited expression of MUC1 in response to *tcpA* can be attributed to non-invasive nature of bacterium.

It has been suggested that MUC protein interacts with bacterial adhesins through mucin oligosaccharide side chains; however, it entraps bacteria and prevents enterocyte from damage when overexpressed [4, 7, 21]. Therefore, it seems that the cooperative or restrictive role of mucin in bacterial pathogenesis is dose-dependent and relies on different stages of bacterial infection. As an enteric pathogen, *V. cholerae* possesses several strategies to overcome the physical mucus barrier of intestinal tract and induce the immune system. It seems that *V. cholerae* has a dual function in its interaction with mucins, i) the bacterium penetrates to intestinal mucus layer by flagellum, cleaves mucin proteins and facilitates bacterial movement probably via HA/P (hemagglutinin/protease) and contribute to bacterium dissemination, ii) promotes microcolony formation by induction of increased MUC2 and MUC3 mucins secretion.^{2,5} However, the production of mucinase by bacteria which helps to overcome the restrictive role of mucin should not be ignored.

TLR4 induces inflammatory cascade, through NF- κ B pathway, in response to LPS of Gram-negative bacteria and plays essential role in host defense through production of TNF- α and iNOS by immune cells [22, 23]. In the current study, rTcpA moderately stimulated expression of *tlr4* gene in Caco-2 model in a dose-dependent manner which is supposed to play a mucosal protective role against bacterial infections in baseline concentration and may trigger an abandoned inflammatory response when overproduced by lamina propria mononuclear and intestinal epithelial cells [24]. This proposes the TcpA protein as a promising candidate for prophylaxis applications.

In this study, the expression of *tlr1* was up-regulated by rTcpA. TLR1 recognizes peptidoglycan and triacyl-lipoproteins in concert with TLR2 (as a heterodimer) in Gram-negative and Gram-positive bacteria and promotes the activation of NF- κ B, and cytokines production by DCs and inflammatory IFN- γ T-cells (Th1). IFN- γ enhances antigen processing and presentation and displays immunomodulatory

effects, boosting the anti-microbial responses [25, 26] . It seems that TcpA induces *tlr1* overexpression and probably improves Caco-2 cell protection.

No significant increase was observed in NOD2 expression in our study which may be one reason for moderate grade inflammation of cholera. NOD1 and NOD2 are specialized NOD-like receptors that contribute to the recognition of some pathogenic microorganisms that can invade and multiply intracellularly. Considering the extracellular nature of *V. cholerae*, no change in NOD2 can be attributed to bacterial lifestyle [27] .

Conclusions

In conclusion, TcpA protein of *V. cholerae* i) has a statistically significant effect on the expression of *muc* genes in a dose-dependent manner. ii) induces the overexpression of mucin genes by intestinal epithelial cells which could increase their protection versus invasive intestinal pathogens, iii) moderately stimulates expression of *tlr4* and overexpresses *tlr1*, both of which together are supposed to help the promising protection against *V. cholerae* infection in prophylaxis applications, IV) no change in NOD2 expression might be attributed to the non-invasive nature of *V. cholerae* as an intestinal pathogen.

Methods

Ethics

This study was approved by the Medical Ethics Committee of Tarbiat Modares University (IR.TMU.REC.1395.339).

Cloning, expression, and purification of rTcpA

Bacterial strains

Vibrio cholerae (ATCC 14035) and *Escherichia coli* BI-21 (DE3) were obtained from the archive of our laboratory in Department of Bacteriology, Tarbiat Modares University, Tehran, Iran, and after their cultivation on Sheep blood agar were used for amplification and production of rTcpA.

Amplification of *tcpA* using PCR technique

Genomic DNA was extracted from *V. cholerae* (ATCC 14035) and used for *tcpA* gene amplification by forward and reverse primers specifically chosen according to whole-genome sequence of *V. cholerae* N16961 (Genbank accession number CP028827) Forward: 5'-GCTGGATCCATGACATTACTCGAAGTGATCATC-3' and Reverse: 5'-GCTCTCGAGGCTGTTACCAAATGCAACGCCGAA-3'. PCR conditions were as follows: initial denaturation at 95°C for 5 min, secondary denaturation at 95°C for 30sec, annealing at 53°C for 30sec, extension at 72°C for 1min and a final extension at 72°C for 2 min. The amplification was performed for 35 cycles.

Purification of PCR products and cloning

PCR product from *tcpA* amplification was purified using the Spin Combo kit (Biotech, Chinese) from agarose gel and digested with *Bam*HI and *Xho*I (Thermo Fisher Scientific, USA). The expression vector, pET-28a, was phosphorylated by rSAP (Thermo Fisher Scientific, USA) and ligated with the purified *tcpA* gene using T4 DNA ligase (Thermo Fisher Scientific, USA). The *tcpA*-pET-28a construct was transformed into *E.coli* BL-21 and colony PCR and plasmid extraction was performed to confirm the ligation. The ligated vector was sequenced (BIONEER company, South Korea) to confirm the identity of cloned gene.

Expression and purification of rTcpA

E.coli BL-21 was transformed with pET-28a for the production of rTcpA. Bacteria were grown in the LB broth (Thermo Fisher Scientific, USA) containing 50 µg/mL kanamycin for overnight at 37 °C and then diluted into new LB broth and incubated again at 37 °C with shaking at 230 rpm until the achievement to OD=0.6 at λ=600 nm. Bacterial culture was treated with 0.01 mM IPTG for induction of *tcpA* expression and further grown at 30 °C with shaking at 230 rpm (the concentration of IPTG, incubation temperature and shaking speed were set up in separate assays).

Subsequently, bacterial cells were lysed by sonication and supernatant was collected for analysis by SDS-PAGE [28]. The rTcpA protein was purified using Ni-column chromatography according to worksheet protocol (CM Sephadex C-25GE; Healthcare Life Science). The concentration of purified protein was determined using BCA protein assay kit (Thermo Fisher Scientific, USA).

Confirmation of rTcpA identity by western immunoblotting

The identity of the purified rTcpA was analyzed by western blotting (WB) assay. Briefly, the SDS-PAGE gel was electroblotted onto polyvinylidene difluoride (PVDF) membrane and the blotted PVDF was blocked with 1% skim milk for overnight at 4 °C. After washing, membranes were soaked in 1:1000 diluted anti-polyhistidine antibody (Abcam, Cambridge, USA) at 4 °C for 24 h. After incubation with HRP conjugated rabbit anti-IgG (Abcam, USA) at room temperature (RT), WB-enhanced chemiluminescence (ECL) substrate was added to the membrane for chemiluminescence detection. The membrane was then lifted and the photoluminescence recorded by ECL camera.

Evaluation of rTcpA cytotoxicity on Caco-2 cell line

Human colon carcinoma cell line (Caco-2 cells) were purchased from Pasteur Institute of Iran and cultured in Dulbecco's Modified Eagle Media (DMEM) F12 (Gibco, Thermo scientific, USA) containing L-glutamine and high glucose, supplemented by 10% fetal bovine serum (Gibco, Thermo scientific, USA) and 1% penicillin-streptomycin. The cell culture flasks were incubated at 37 °C with 5% CO₂ in a moist atmosphere [29]. The Caco-2 cells start to polarize when confluence and macromolecules are sorted and

maintained between apical and basolateral surfaces of confluent cells. Moreover, markers of colonocytes are also present in Caco-2 cells [30].

The Caco-2 cells were trypsinated when reached 90% confluency and seeded in 96-well plates (SPL life science, South Korea) at a density of 2×10^4 cells per well and incubated at 37 °C with 5% CO₂. The confluent monolayer was treated with different concentrations of rTcA (0.1, 1 and 10 µg/mL) for 24, 48 and 72 h. Subsequently, 50 µL of the medium was removed and replaced with MTT (Methyl Thiazolyl diphenyl-tetrazolium bromide) reagent. After 4 h incubation, 100 µL DMSO was added, mixed well and the absorbance was read at 570 nm and the cell viability was measured by the following formula:

Cell viability (%) = OD of sample/OD of control $\times$ 100 [31].

Caco-2/ Peripheral Blood Mononuclear Cells (PBMCs) co-culture

PBMCs were separated from whole blood of healthy volunteers by Ficoll-Hypaque centrifugation method (400g, 20 min) [32, 33]. The collected PBMCs were washed 2 times with PBS and purified PBMCs were seeded in DMEM supplemented by 10% inactive fetal bovine serum and 1% penicillin-streptomycin at 37 °C with 5% CO₂ (28). The Caco-2 cells were transferred at a density of 2.5×10^5 cell/mL into a T25 tissue culture flask and 5 mL of complete medium containing DMEM F12, FBS 10%, and penicillin-streptomycin was added with density of 2×10^6 cell/mL of freshly isolated PBMCs [34].

Determination of TLRs, NODs, and *mucin* related genes expression in Caco-2/PBMCs co-culture treated with rTcA by real-time PCR

RNA extraction and cDNA synthesis

The Caco-2/PBMCs co-culture was treated by different concentrations of naked rTcA (1, 5, 10 and 50 µg/mL). The Caco-2/PBMCs co-cultures without treatment were used as control. The treatment was performed on co-culture model for 24h at 37 °C in CO₂ atmosphere and Caco-2 cells were collected and lysed by trizol reagent (Sigma Aldrich, Germany) for RNA extraction. Accordingly, chloroform was added to the cell lysate and centrifuged (15min, 1200g and 4 °C), afterward the aqueous phase was mixed with 2-propanol and centrifuged. The isolated total RNA was washed twice with ethanol 70% and after drying was solubilized in diethyl pyrocarbonate (DEPC) treated water. The purity of RNA was determined by absorbance at OD_{260/280} and only samples with a ratio of 1.8 ± 2.0 were subjected for cDNA synthesis by cDNA Synthesis Kit (YektaTajhizAzma, IRAN). According to manufacturer protocol, 100 ng template RNA, 50 µM Random hexamer, 50 µM Oligo dT and DEPC treated water were mixed and incubated for 5 min at 70°C, after which M-MLV (Moloney Murine Leukemia Virusreverse transcriptase) (10,000 U), dNTP 10mM, RNasin (40u/µl), 5X first-strand buffer were added and the mixture was incubated for 60 min at 37 °C. The reaction was terminated by heating at 37 °C for 5 min.

Real-time PCR analysis

Real-time PCR was performed to determine the probable role of rTcPA in the modulation of the TLRs, NODs, and *mucin* related genes expression (*muc1,3and4*) using the Applied Biosystems 7500 (Thermo Fisher Scientific, USA). The qPCR was performed according to the following conditions: Pre-incubation at 95°C for 5 min, 45 cycles consisting of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec. The melting pick analysis was performed in 95 °C for 5 min. Each PCR reaction contained 10µL 5x Real-time PCR Master Mix (Takara, Japan), 2 µL cDNA template, 0.8µL of each primer and 6.4µL distilled water in a total reaction volume of 20 µL. The nucleotide sequence of the forward and reverse primers used for amplification of *muc* (*muc1, 3and 4*), *tlr* (*tlr1, 4*) and *nod* genes (*nod1, nod2*) are depicted in Table 2. In each reaction, the same amounts of RNA were converted into cDNA and pipetting error was removed. The expression of GAPDH gene was considered as the internal control for each sample and the ΔCT (CT target–CT reference) of each sample and expression fold change were calculated. Each real-time PCR reaction was carried out in triplicate and results were presented as Mean $\pm$ SD.

Statistical Analysis

The Kruskal-Wallis test was used to perform statistical analysis (using SPSS software,ver. 16) to compare the gene expression between treated Caco-2/PBMC co-culture cells groups with untreated cells and a p-value <0.05 was considered as significant. The correlation coefficient between the rTcPA concentration and the different genes expression level was determined by Spearman test.

Declarations

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

MG has performed all the laboratory tests, also has major contribution in drafting the manuscript. BB has supervised and funded the study entirely and has participated in writing the results and conclusions. SN has a major role in analysis of the data as well as drafting the manuscript.

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Ethics approval

This study was approved by the Medical Ethics Committee of Tarbiat Modares University (IR.TMU.REC.1395.339).

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing of interest.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1. Al-Shukri MSM, Abdul-Lateef AL. Molecular detection of cholera toxin genes in *Vibrio cholerae* infection in human. *J Chem Pharm Sci*. 2017; 10(2): 972-79.
2. Almagro-Moreno S, Pruss K, Taylor RK. [Intestinal colonization dynamics of *Vibrio cholerae*](#). *PLoS Pathog*. 2015;11(5):1004787.
3. van Putten JPM, Strijbis K. [Transmembrane Mucins: Signaling Receptors at the Intersection of Inflammation and Cancer](#). *J Innate Immun*. 2017;9(3):281-299.
4. Cornick S, Tawiah A, Chadee K. [Roles and regulation of the mucus barrier in the gut](#). *Tissue Barriers*. 2015;3(1-2):982426 .
5. Bhowmick R, Ghosal A, Das B, Koley H, Saha DR, Ganguly S, et al. [Intestinal adherence of *Vibrio cholerae* involves a coordinated interaction between colonization factor GbpA and mucin](#). *Infect Immun*. 2008;76(11):4968-77.
6. Dharmani P, Srivastava V, Kissoon-Singh V, Chadee K. [Role of intestinal mucins in innate host defense mechanisms against pathogens](#). *J Innate Immun*. 2009;1(2):123-35.
7. Hanisch FG, Müller S. [MUC1: the polymorphic appearance of a human mucin](#). *Glycobiology*. 2000;10(5):439-49.
8. McAuley JL, Linden SK, Png CW, King RM, Pennington HL, Gendler SJ, et al. [MUC1 cell surface mucin is a critical element of the mucosal barrier to infection](#). *J Clin Invest*. 2007;117(8):2313-24.

9. Queen J, Satchell KJ. [Neutrophils are essential for containment of *Vibrio cholerae* to the intestine during the proinflammatory phase of infection.](#) Infect Immun. 2012;80(8):2905-13.
10. Bandyopadhyaya A, Chaudhuri K. Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) signaling during *Vibrio cholerae* infection. MOJ Immunol; 2015;2(1):1-8.
11. Behzadi E, Behzadi P. [The role of toll-like receptors \(TLRs\) in urinary tract infections \(UTIs\).](#) Cent European J Urol. 2016;69(4):404-410.
12. de Zoete MR, Flavell RA. [Interactions between Nod-Like Receptors and Intestinal Bacteria.](#) Front Immunol. 2013;4:462.
13. Takeda K, Akira S. [Toll-like receptors in innate immunity.](#) Int Immunol. 2005;17(1):1-14.
14. Neutra MR, Frey A, Kraehenbuhl JP. [Epithelial M cells: gateways for mucosal infection and immunization.](#) Cell. 1996;86(3):345-8.
15. Krebs SJ, Taylor RK. [Protection and attachment of *Vibrio cholerae* mediated by the toxin-coregulated pilus in the infant mouse model.](#) J Bacteriol. 2011;193(19):5260-70.
16. Chaturvedi P, Singh AP, Batra SK. [Structure, evolution, and biology of the MUC4 mucin.](#) FASEB J. 2008;22(4):966-81.
17. Dong W, Zong Q, Gan L, Qin W, Zhu G, Bao W, et al. Age-dependent expression of MUC4 and MUC13 gene in suta (DUROC×MEISHAN) PIGLETS. J Sci. 2015;5(11) :1024-28.
18. Vieira MA, Gomes TA, Ferreira AJ, Knöbl T, Servin AL, Liévin-Le Moal V. [Two atypical enteropathogenic *Escherichia coli* strains induce the production of secreted and membrane-bound mucins to benefit their own growth at the apical surface of human mucin-secreting intestinal HT29-MTX cells.](#) Infect Immun. 2010;78(3):927-38.
19. Li X, Bleumink-Pluym NMC, Luijckx YMCA, Wubbolts RW, van Putten JPM, Strijbis K. [MUC1 is a receptor for the *Salmonella* SiiE adhesin that enables apical invasion into enterocytes.](#) PLoS Pathog. 2019;15(2):e1007566.
20. Sheng YH, Triyana S, Wang R, Das I, Gerloff K, Florin TH, et al. [MUC1 and MUC13 differentially regulate epithelial inflammation in response to inflammatory and infectious stimuli.](#) Mucosal Immunol. 2013;6(3):557-68.
21. Lindén SK, Florin TH, McGuckin MA. [Mucin dynamics in intestinal bacterial infection.](#) PLoS One. 2008;3(12):1-14.
22. Branger J, Knapp S, Weijer S, Leemans JC, Pater JM, Speelman P, et al. [Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice.](#) Infect Immun. 2004;72(2):788-94.
23. Royle MC, Töttemeyer S, Alldridge LC, Maskell DJ, Bryant CE. [Stimulation of Toll-like receptor 4 by lipopolysaccharide during cellular invasion by live *Salmonella typhimurium* is a critical but not exclusive event leading to macrophage responses.](#) J Immunol. 2003;170(11):5445-54.
24. Frosali S, Pagliari D, Gambassi G, Landolfi R, Pandolfi F, Cianci R, et al. How the intricate interaction among Toll-Like Receptors, microbiota, and intestinal immunity can influence gastrointestinal pathology. J Immunol Res. 2015;2015:489821.

25. Cooper AM, Khader SA. [IL-12p40: an inherently agonistic cytokine](#). Trends Immunol. 2007;28(1):33-8.
26. Kak G, Raza M, Tiwari BK. [Interferon-gamma \(IFN- \$\gamma\$ \): Exploring its implications in infectious diseases](#). Biomol Concepts. 2018;9(1):64-79.
27. Moreira LO, Zamboni DS. [NOD1 and NOD2 Signaling in Infection and Inflammation](#). Front Immunol. 2012;3:328.
28. Rollenhagen JE, Kalsy A, Cerda F, John M, Harris JB, Larocque RC, et al. [Transcutaneous immunization with toxin-coregulated pilin A induces protective immunity against *Vibrio cholerae* O1 El Tor challenge in mice](#). Infect Immun. 2006;74(10):5834-9.
29. Hamzeloo-Moghadam M, Taiebi N, Mosaddegh M, Tehrani E B, Esmaeili S. The effect of some cosolvents and surfactants on viability of cancerous cell lines. Res J Pharmacogn. 2014;1 (3):41-45.
30. Engle MJ, Goetz GS, Alpers DH. [Caco-2 cells express a combination of colonocyte and enterocyte phenotypes](#). J Cell Physiol. 1998;174(3):362-9.
31. Bahrami B, Macfarlane S, Macfarlane GT. [Induction of cytokine formation by human intestinal bacteria in gut epithelial cell lines](#). J Appl Microbiol. 2011;110(1):353-63.
32. Fang HW, Fang SB, Chiang Chiau JS, Yeung CY, Chan WT, Jiang CB, et al. Inhibitory effects of *Lactobacillus casei* subsp. rhamnosus on *Salmonella* lipopolysaccharide-induced inflammation and epithelial barrier dysfunction in a co-culture model using Caco-2/peripheral blood mononuclear cells. J Med Microbiol. 2010;59(5):573-9.
33. Nicole Seeligmüller. Faster Isolation of PBMC Using Ficoll-Paque. Plus in the Eppendorf® Centrifuge 5920 R. Application Note 372 June 2016.
34. D. Haller, C.Bode, W. P. Hammes, A. M. A Pfeifer, E. J .Schivrin, S. Blum. Non-Pathogenic Bacteria Elicit a Differential Cytokine Response by Intestinal Epithelial Cell/leucocyte Co-Cultures. Gut. 2000;47(1):79-87.
35. Mitas M, Mikhitarian K, Walters C, Baron PL, Elliott BM, Brothers TE, et al. [Quantitative real-time RT-PCR detection of breast cancer micrometastasis using a multigene marker panel](#). Int J Cancer. 2001;93(2):162-71.
36. Pan Q, Tian Y, Li X, Liu Y, Song L, Yang Y, et al. [Enhanced membrane-tethered mucin 3 \(MUC3\) expression by a tetrameric branched peptide with a conserved TFLK motif inhibits bacteria adherence](#). J Biol Chem. 2013;288(8):5407-16.
37. Damera G, Xia B, Sachdev GP. [IL-4 induced MUC4 enhancement in respiratory epithelial cells in vitro is mediated through JAK-3 selective signaling](#). Respir Res. 2006;7:39.
38. Furrie E, Macfarlane S, Thomson G, Macfarlane GT. [Toll-like receptors-2, -3 and -4 expression patterns on human colon and their regulation by mucosal-associated bacteria](#). Immunology. 2005;115(4):565-74.
39. Geng HL, Lu HQ, Zhang LZ, hang H, Zhou L, Wang H, et al. [Increased expression of Toll like receptor 4 on peripheral-blood mononuclear cells in patients with coronary arteriosclerosis disease](#). Clin Exp Immunol. 2006;143(2):269-73.

40. Navarro R, Delgado-Wicke P, Nuñez-Prado N, Compte M, Blanco-Toribio A, et al. [Role of nucleotide-binding oligomerization domain 1 \(NOD1\) in pericyte-mediated vascular inflammation](#). J Cell Mol Med. 2016;20(5):980-6.
41. King AE, Horne AW, Hombach-Klonisch S, Mason JI, Critchley HO. [Differential expression and regulation of nuclear oligomerization domain proteins NOD1 and NOD2 in human endometrium: a potential role in innate immune protection and menstruation](#). Mol Hum Reprod. 2009;15(5):311-9.
42. Kanellou P, Zaravinos A, Zioga M, Stratigos A, Baritaki S, Soufla G, et al. [Genomic instability, mutations and expression analysis of the tumour suppressor genes p14\(ARF\), p15\(INK4b\), p16\(INK4a\) and p53 in actinic keratosis](#). Cancer Lett. 2008;264(1):145-61.

Tables

Table 1 The expression of *muc*, *tlr* and *nod* genes when treated by different concentration of rTcpA (µg/mL)

gene	Expression rate (Fold changes) in different concentrations of rTcpA (µg/mL)				Sig.
	1(µg/mL)	5(µg/mL)	10(µg/mL)	50(µg/mL)	
<i>muc1</i>	2.3	5.5	7.5	8.6	<0.05
<i>muc3</i>	1.3	7.6	12.2	76.8	<0.05
<i>muc4</i>	3.8	9.5	14.9	185	<0.05
<i>tlr1</i>	1.01	1.93	3.94	30.73	<0.05
<i>tlr4</i>	0.309	0.564	1.16	4.962	<0.05
<i>nod1</i>	0.177	0.303	0.33	0.37	
<i>nod2</i>	0.555	2.35	4.36	19.904	<0.05

muc (*muc1*, *muc3*, *muc4*), *tlr* (*tlr1*, 4) and *nod* (*nod 1*, 2) genes

Table 2 The sequence of oligonucleotide primers used for Real-time PCR.

Gene	Forward primer	Reverse primer	Reference
Mucin 1	5'-ACCATCCTATGAGCGAGTACC-3'	5'-ACCATCCTATGAGCGAGTACC-3'	[35]
Mucin 3	5'-CCGGACCTCAATGACAACACT-3'	5'-CCGGACCTCAATGACAACACT-3'	[36]
Mucin4	5'-GCCCAAGCTACAGTGTGACTCA-3'	5'-ATGGTGCCGTTGTAATTTGTTGT-3'	[37]
TLR1	5'-GAAGATTTCTTGCCACCCTAC-3'	5'- GAACACAATGTGCAGACTCTC-3'	[38]
TLR4	5'-TGGAAGTTGAACGAATGGAATGTG-3'	5'-ACCAGAACTGCTACAACAGATACT-3'	[39]
NOD1	5'- AAGCGAAGAGCTGACCAAATAC-3'	5'-TCCCAGTTTAAGATGCGTGAG-3'	[40]
NOD2	5'- GCCACGGTGAAAGCGAAT-3'	5'- GGAAGCGAGACTGAGCAGACA-3'	[41]
GAPDH	5'-GGAAGGTGAAGGTCGGAGTCA-3'	5'-GTCATTGATGGCAACAATATCCACT-3'	[42]

Figures

Figure 1

SDS page of rTcA, lane 1: whole cell proteome, lane 2: supernatant of cells, lane 3: flow through, lanes 4-8: elution 1-5 of rTcA, respectively, lane 9: Western blotting of rTcA by anti-polyhistidine antibody and M: protein molecular marker.