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Gene Expression Profiling of the Human Intestinal Epithelial Innate Immune Response to Two Major *Vibrio* Pathogens

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Abstract

Diarrheal diseases caused by bacteria, viruses, and parasites rank fourth in fatality rate among all other diseases around the globe, causing at least 3 million deaths a year. *Vibrio cholerae* and *V. parahaemolyticus* are two major pathogens that elicit diarrheal related diseases through very different mechanisms. The goal of this investigation is to compare and expand our knowledge of the innate immune response to these *Vibrio* pathogens. As such, the human intestinal epithelial cell line, Caco-2, was challenged for 2 hours with the two *Vibrio* species, and mRNA expression of 84 different genes involved in inflammation or autoimmune disease was quantified using a pathway focused real time reverse transcription PCR array. Appropriate controls for the quantitative PCR for genomic DNA contamination and analyses of housekeeping genes are included in these arrays. We found both *V. cholerae* and *V. parahaemolyticus* most notably induce over expression (10-fold or higher) of the following cytokines and chemokines; TNF-α, IL-7, CXCL-1 (GRO-α), CXCL-2 (GRO-β), CXCL-10 (IP-10), CCL-2 (MCP-1) and CXCL-11 (I-TAC). Of the expected proinflammatory cytokines TNF-α was significantly increased (over 50 fold) whereas IL-1 and IL-6 were only increased by about 4 fold. In addition, many neutrophil chemoattractants (CXCL family) were upregulated, but not IL-8. We were surprised and intrigued by the high level (more than 50 fold) of IL-7 gene expression induced by both species as IL-7 is generally thought of as a hematopoietic cytokine. Because *V. parahaemolyticus* causes inflammatory diarrheal disease and *V. cholerae* causes noninflammatory diarrhea, we were particularly interested in differences in gene expression between the two. The most notable of these was the induction of LIGHT (TNFSF14) observed in *V. parahaemolyticus* but not *V. cholerae*. In summary, we observed a many proinflammatory cytokines and chemokines upregulated by both *Vibrio* species in Caco-2 cells. We will use these array data to guide further investigations confirming our initial observations and probing the functional significance of these inflammatory pathways in both species.

Introduction

- Approximately 1 to 1.5 billion diarrheal infections have been attributed to enterotoxin producing bacteria. Diarrhea diseases caused by bacteria, viruses, and parasites ranks fourth in fatality rate among all other diseases around the globe causing over 3 million deaths a year, and has a major impact on developing and low income countries. *Vibrio cholerae* and *Vibrio parahaemolyticus* have been found to be two major elicitors of diarrhea and diarrhea related diseases.
- Illnesses due to *Vibrio parahaemolyticus* typically result from the consumption of contaminated, raw, or under-cooked shellfish within 24 hours of pathogen ingestion. Traditionally the virulence factor thought to be responsible for the symptoms has been thermostable direct hemolysin (tdh); however, full understanding of the organism's pathogenesis remains unclear. *V. parahaemolyticus* can be found in estuarine and temperate coastal marine waters throughout the world. The organism can be found both free living or as commensals with reservoir hosts including fish and shellfish. Proper cooking by the consumer could eliminate the chance of contracting the disease. Undercooked oysters prior to consumption along with warmer global water temperatures has led to the re-emergence of this food pathogen.
- *Vibrio cholerae* infection can result from the consumption of contaminated water or food sources. Some strains of the bacterium produce the enterotoxin known as Cholera Toxin, which is the major cause of cholera symptoms. Figure 1 shows typical *V. cholerae* organisms.

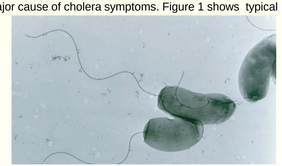
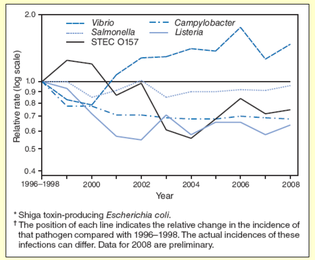


Figure 1. Image of *Vibrio cholerae*. Cholera bacteria have a single polar flagellum and can live as free-swimming individuals or form colonies called biofilms.



* Shiga toxin-producing *Escherichia coli*.
† The position of each line indicates the relative change in the incidence of that pathogen compared with 1996–1998. The actual incidences of these infections can differ. Data for 2008 are preliminary.

Figure 2. Emergence of *Vibrio*. Among common foodborne pathogens in the United States, incidence of *Vibrio* (mostly *V. parahaemolyticus*) associated infection shows an upward trend. (reprint from CDC/MMWR)

Methods

- The human intestinal epithelial cell line Caco-2 (HTB-37) was used and cells were grown in ATCC-formulated Eagle's Minimum Essential Medium supplemented with 20% fetal bovine serum and kept under 5% CO2 and at 37°C. For the experiments the cells were seeded into T-25 flasks at a density of 2.52 X 10^5 cells/mL. The cells were grown to 100% confluency and challenged with *V. cholerae* or *V. parahaemolyticus* at MOI of 100 (approximately 1.0 X 10^6 CFU).
- Before the challenge Caco-2 cells were washed once with PBS and fresh MEM only was added. *V. cholera* or *V. parahaemolyticus* from an overnight culture was concentrated, washed, and resuspended in fresh MEM and added to each T-25 flask for 2 hours. For the control experiments all procedures were followed except for bacterial challenge. The 2 hour challenge was chosen based on results collected from conventional PCR for genes TNF, IL-1, and GAPDH. For conventional PCR the total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, CA) following manufactures recommendations. The mRNA was reversed transcribed to cDNA using oligo(dT)20 primers following the manufactures instructions using the SuperScript™ III First-Strand Synthesis System (Invitrogen, CA). The primers used are shown in Table 1. The products were then run on a 2% agarose gel and visualized as seen in Figure 4.
- The total RNA used for the RT² PCR Profiler Array was isolated after 2 hours from Caco-2 cells using RT² qPCR-Grade™ RNA Isolation Kit (SABiosciences, MD) by following the manufacturer's instructions. RQ1 RNase-Free DNase (Promega, CA) was added during the on column DNA removal step and also after the RNA isolation following the manufacturers recommendations. The RNA quality was determined by gel electrophoresis. RNA concentration was determined using Quanti-iT™ RNA Assay Kit (Invitrogen, CA) to ensure an equal amount of RNA was used for cDNA synthesis. Total RNA was converted to cDNA using RT² First Strand Kit (SABiosciences, MD). The cDNA was added with 2X Formulation RT² SYBR Green / ROX PCR Master Mix for ABI Instrumentation (SABiosciences, MD) and other reagents according to manufactures recommendations. Twenty four microliters of cDNA was loaded into a RT² Profiler PCR Array Human Inflammatory Response & Autoimmunity plate and used for relative quantification with the Applied Biosystems Real-Time PCR 7300 System used according to the manufacturer's protocol.

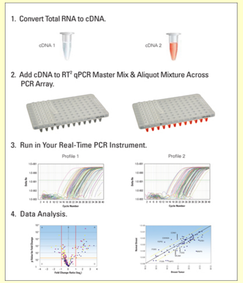


Figure 3. RT² Profiler™ PCR Array System. Description of the PCR array procedure from SA Biosciences.

Table 1. Primers used for reverse transcriptase PCR. In order to determine the optimal MOI and length of challenge initial experiments were done to measure expression of TNF and IL1.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
TNF[a]	GGCCCAGGCAGTCAGATCAT	GGGGCTCTTGATGGCAGAGA
IL1[a]	TTCCCTGCCCACAGACCTTC	AGGCCCAAGGCCACAGGAT
GAPDH[b]	CCACCCATGGCAAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC

[a] TNF, Tumor Necrosis Factor
[a] IL-1, Interleukin 1
[b] GAPDH, glyceraldehydes-3-phosphate Dehydrogenase

Results

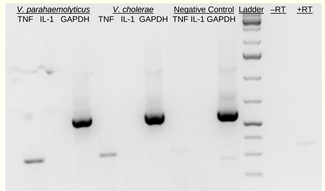


Figure 4. Gene Expression of TNF, IL-1, and GAPDH after 2 hour challenge. Intestinal epithelial cells, Caco-2 were challenged at an MOI of 100 for 2 hours with *V. parahaemolyticus*, *V. cholerae*, and negative control with no bacteria. Cell were lysed, RNA was isolated, and gene expression was determined by reverse transcriptase PCR.

Table 2. Fold regulation of similar genes by both *Vibrio* Species. Caco-2 cells, 2 hour challenge with *V. parahaemolyticus* and *V. cholerae,* showing genes with greater than 4 fold upregulation when compared to the control. All samples were done in duplicate and standardized against the control using housekeeping genes. Average standard deviation between the two *V. parahaemolyticus* and *V. cholerae* arrays were 0.76 ct was 0.53 ct respectively. Average standard deviation between the two control arrays was 1.0 ct.

Gene Symbol	Gene Name	Genebank	Vp Fold Regulation	Vc Fold Regulation
Tnf	Tumor necrosis factor (TNF superfamily, member 2)	NM_000594	**57.08**	**73.31**
Il7	Interleukin 7	NM_008371	**64.00**	**56.34**
Cxcl1	Chemokine (C-X-C motif) ligand 1	NM_001511	24.59	29.57
Cxcl2	Chemokine (C-X-C motif) ligand 2	NM_002089	25.63	28.46
Cxcl10	Chemokine (C-X-C motif) ligand 10	NM_001565	18.77	27.78
Ccl2	Chemokine (C-C motif) ligand 2	NM_002982	23.92	15.15
Cxcl11	Chemokine (C-X-C motif) ligand 11	NM_019494	22.16	21.72
Ltb	Lymphotoxin B	NM_008518	10.48	9.65
Flt3l	Fms-related tyrosine kinase 3 ligand	NM_001459	5.88	6.03
Il22	Interleukin 22	NM_020525	5.28	4.55
Il1rap	Interleukin 1 receptor accessory protein	NM_002182	4.07	4.5

Vp, *Vibrio parahaemolyticus*
Vc, *Vibrio cholerae*

Table 3. Fold regulation of Caco-2, 2 hour challenge with *Vibrio parahaemolyticus*. Genes with greater than 4 fold upregulation when challenged with *V. parahaemolyticus* but not *V. cholerae*. All samples were done in duplicate and standardized against the control using housekeeping genes.

Gene Symbol	Gene Name	Genebank	Fold Regulation
Tnfsf14	Tumor necrosis factor (ligand) superfamily, member 14	NM_003807	**14.07**
Ccl11	Chemokine (C-C motif) ligand 11	NM_002986	6.73
Il6	Interleukin 6 (interferon, beta 2)	NM_000600	5.62
Ccl20	Chemokine (C-C motif) ligand 20	NM_016960	4.99
Tlr4	Toll-like receptor 4	NM_138554	4.89
Ccl25	Chemokine (C-C motif) ligand 25	NM_009138	4.66
Ccl1	Chemokine (C-C motif) ligand 1	NM_011329	4.41
Itgb2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	NM_000211	4.29
Ccl4	Chemokine (C-C motif) ligand 4	NM_002984	4.11
Ccl5	Chemokine (C-C motif) ligand 5	NM_002985	4.08

Table 4. Fold regulation of Caco-2, 2 hour challenge with *Vibrio cholerae*. Genes with greater than 4 fold upregulation when challenged with *V. cholerae* but not *V. parahaemolyticus*. All samples were done in duplicate and standardized against the control using housekeeping genes.

Gene Symbol	Gene Name	Genebank	Fold Regulation
Csf1	Colony stimulating factor 1 (macrophage)	NM_007778	6.44
C3ar1	Complement component 3a receptor 1	NM_004054	5.45
Il22	Interleukin 22	NM_020525	4.55
C3	Complement component 3	NM_009778	4.53
Ccl8	Chemokine (C-C motif) ligand 8	NM_021443	4.4

Conclusions

Over expression of Tumor Necrosis Factor (TNF) in *V. parahaemolyticus* and *V. cholerae* challenge

TNF was shown to be over expressed in Caco-2 cells by both pathogens using conventional PCR and PCR array. TNF acted as an internal positive control and by showing over expression we can be confident we were able to simulate an infection. TNF is produced by a variety of cell types and acts as a key mediary in the local inflammatory immune response. TNF is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection.

Over expression of Interleukin 7 (IL-7) in *V. parahaemolyticus* and *V. cholerae*

IL-7 over expression by Caco-2 cells in response to both pathogens was a surprising result which has not previously been reported. IL-7 appears to be an important cytokine in the human immune response to *Vibrio* pathogens. IL-7 is a stromal cell-derived cytokine essential for the development and differentiation of B and T lymphocytes. IL-7 is capable of activating monocytes/macrophages and natural killer (NK) cells. For these reasons, IL-7 is a potential immunotherapeutic because of its potent capacity to amplify T-cell based immunity. Further studies could investigate the administration of IL-7 after *Vibrio* pathogen infection to potentially increase the recovery period.

Over expression of Tumor necrosis factor superfamily, member 14 (LIGHT) in *V. parahaemolyticus* but not *V. cholerae* challenge

While LIGHT was shown to be over expressed more than 14-fold in the *V. parahaemolyticus* challenge, it was not notability over expressed in the *V. cholerae* challenge. LIGHT is a member of the tumor necrosis factor superfamily that contributes to the regulation of immune responses. LIGHT can influence T-cell activation by engaging cell surface receptors of T cells and also other cells which engages pathways to fight cancer and infectious disease. Although both species of *Vibrio* seemed to induce a similar immune response, LIGHT is interesting because it was only over expressed in the *V. parahaemolyticus* challenge indicating an alternative immune response to *V. cholerae*.

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