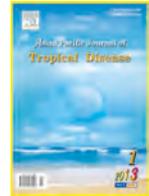




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# CT gene modulate differential expression of chitinase gene under variant habitats in *Vibrio cholerae*

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## PEER REVIEW

## ABSTRACT

**Peer reviewer**

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**Comments**

The adaptation of *vibrio* for utilization of chitin not just only for carbon source but it also provides ideal support for adherence that is essential for biofilm formation.

**(Details on Page 24)**

**Objective:** To investigate the interrelation of cholera toxin gene (CT gene) in expression of chitinase gene under different pH conditions among pathogenic and Non-pathogenic strains of *Vibrio cholera* (*V. cholera*). **Methods:** The chitinase assay well diffusion method and calorimetric chitinase assay were performed. Further, time depended chitinase activity among pathogenic and nonpathogenic strain was evaluated with control as *Escherichia coli*. The expressed protein in variant environment was purified by cascade of chromatographic techniques. The partially purified protein was analyzed by SDS-PAGE in both the strain of *V. cholera*. **Results:** The results have shown differential expression of chitinase gene among *vibrio* in time depended chitinase activity, purification of expressed protein and SDS-PAGE analysis. **Conclusions:** From the current study, two conclusions came in picture, habitat is prime factor that regulation of chitin gene expression among many bacterial strains, second, moreover among the *vibrio* pathogenic strains (CT+) expression of chitinase gene is more precisely regulated by CT gene rather than external environments while in non-pathogenic strain (CT-) completely absent.

## KEYWORDS

Chitinase activity, CT gene, *Vibrio cholera*, Colloidal chitin, Chromatography

## 1. Introduction

*Vibrio cholera* (*V. cholera*), causative agent of waterborne diarrheal disease had known for its genome plasticity and clonal diversity[1]. However, only two among more than 200 O-antigen serogroups are known to date, O1 and O139, cause epidemic cholera outbreaks[2]. It is clear that the acquisition of certain genes encoding virulence factors has enabled certain strains of *V. cholera* to colonize the human intestine and cause disease[3]. The most important of these virulence factors are cholera toxin (CT) that encoded within the genome of the filamentous bacteriophage CT and thus horizontally transferred and the intestinal colonization factor toxin-coregulated pilus (TCP) which encoded in the *Vibrio* pathogenicity island[4]. The acquisition of the *Vibrio*

pathogenicity island, by yet-unclear mechanisms[5], is a critical event in the evolution of epidemic strains of *V. cholera*, since TCP also serves as a receptor for CT[6].

Chitin is one of the most abundant polymers on earth and possibly the most abundant in the aquatic environment, where its association with *V. cholera* has provided the microorganism with a number of advantages, including food availability, adaptation to environmental nutrient gradients, tolerance to stress and protection from predators[7]. Emergent properties of *V. cholera*-chitin interactions occur at multiple hierarchical levels in the environment and include cell metabolic and physiological responses e.g. chemotaxis, cell multiplication, induction of competence, biofilm formation, commensal and symbiotic relationship with higher organisms, cycling of nutrients,

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and pathogenicity for humans and aquatic animals[8]. As factors mediating virulence of *V. cholera* for humans and aquatic animals derive from mechanisms of adaptation to its environment, at different levels of hierarchical scale, *V. cholera* interactions with chitin represent a useful model for examination of the role of primary habitat selection in the development of traits that have been identified as virulence factors in human disease[9].

In the current study primarily we targeted different climatic factors regulate expression of genes and possible interaction of CT and chitinase gene in *V. cholera*. To understand complete etiology of gene expression and interaction we employed various environmental conditions including pH, time of incubation and inducers. We have used *V. cholera* strains with identical genetic makeup with single gene difference and both strain used in this study were wild type. Under variant growth conditions created in controlled environment time dependent chitinase assay both well diffusion and enzymatic was performed with induced and non-induced strains. Further, partial purification of expressed protein was analyzed and validated by SDS-PAGE. The most important factor we focused in our study pH of growth media which play major role in providing adaptation to microbial world to survive in drastic conditions.

## 2. Materials and methods

### 2.1. Bacterial strains

The bacterial strains used in the present study, *V. cholera* strains VC 20 O1 Ogawa and WO5 O1 Inaba was collected from National Institute of Cholera and Enteric Diseases (NICED), Kolkata, West Bengal, India. Bacterial strain *Escherichia coli* (*E. coli*). DH 10 procured from MTCC Chandigarh, India as co-culture. All the bacterial strains used in the current study were in their native wild condition. A brief description provided from NICED Kolkata as *V. cholera* VC20 and WO5 are having only one difference of CT gene. *V. cholera* VC 20 wild type is additionally having cholera toxin gene while WO5 lacks naturally. Other genetic makeup was same for both of *V. cholera* strains.

### 2.2 Colloidal chitin preparation

Ten grams of chitin mixed with 100 mL of concentrated hydrochloric acid and stirred for 24 h at 4 °C. The suspension poured into 5 L of distilled water and centrifuged (12000 r/min for 10 min). The resulting precipitate washed with distilled water until the pH reached 5.0 and then neutralized by addition of 6 N NaOH. The suspension was centrifuged and washed with 3 L of distilled water for desalting (12000 r/min for 10 min). The resulting precipitate suspended with distilled water. The chitin content in the suspension was determined by drying a sample[10].

### 2.3. Colloidal chitin plates preparation

The colloidal chitin plates were prepared as per following compositions, 0.4% colloidal chitin, 0.05% yeast extract, 0.2% di-potassium hydrogen phosphate, 0.1% potassium di-hydrogen phosphate, 0.07% magnesium sulfate pentahydrate, 1.05% sodium chloride, 0.05% potassium chloride, 0.01% calcium chloride and 1.5% agar. The media was

sterilized and plated were prepared[11].

### 2.4. Pretreatment to chitin and inoculation

All the bacterial cultures that have used in this study were grouped in two category; chitin exposed and non-exposed. The bacterial cultures pretreated with chitin flakes in different concentration, different time interval and different source of chitin. Bacterial cultures have grown in optimal condition 37 °C for 24, 48 and 72 h at 150 r/min in orbital incubator shaker.

### 2.5. Chitinase activity test

To evaluate chitinolytic activity among various strains under variant habitat, used in the present study, well diffusion methods was opted. Luria Broth Agar plates containing colloidal chitin used for the determination of chitinase activity. Approximately 6 mm diameters well punched on the plates and 20 µL of samples from overnight cultures stabbed into the mineral salt medium containing 0.2% colloidal chitin. The plates incubated for 2 to 4 d at 37 °C and clear zones around the stabbed site checked for all the strains[12].

### 2.6. Partial purification of chitinase

Chitinase, an extracellular proteolytic enzyme purified with series of chromatographic purification steps from both chitin exposed and non-exposed *V. cholera*. Though Chitinase is extracellular protein, overnight grown bacterial cultures were centrifuged at high-speed rotation 20000 for 15 min to separate bacterial cells from soup. The soup further subjected to the salt precipitation to recover total protein and 70% of ammonium sulphate used to recover proteins[13]. Crude protein was dialyzed to remove salt by dialysis membrane of Molecular Weight Cut Off (MWCO) 14KD for 12 h against 20 mmol phosphate buffer, pH 7.5. The dialyzed samples were loaded in Sephadex-G 200 column. Completely swelled Sephadex-G 200 beads packed under gravity and equilibrated with phosphate buffer. The different fractions eluted with excess of 20 mmol phosphate buffer pH 7.5. The fractions subjected for chitinolytic activity by well diffusion method[14].

### 2.7. Chitinase assay

Colorimetric based chitinase assay performed to evaluate chitin utilization by different bacterial strains under variant conditions. The reaction mixture contained 1ml of 0.1% colloidal chitin in sodium acetate buffer (0.05 mol/L, pH 5.2) and 1 mL culture filtrate was incubated at 37 °C for 2 h in a water bath with constant shaking. Suitable substrate and enzyme blanks were included. Chitinase activity has assayed by the colorimetric method[17]. The reaction has terminated by adding 0.1 mL of 0.08 mol/L potassium tetraborate, pH 9.2 to 0.5 mL of reaction mixture and then boiled in a water bath for 3min. Then 3 mL of diluted p-dimethylaminobenzaldehyde (p-DMAB, Sigma Chemicals Company, USA) reagent added and again incubated at 37 °C for 15 min. The released product in the reaction mixture read at 585 nm in a spectrophotometer (Hitachi, Japan). Chitinase activity was determined using N-acetylglucosamine (Sigma Chemicals Company, USA) as the standard[15–17].

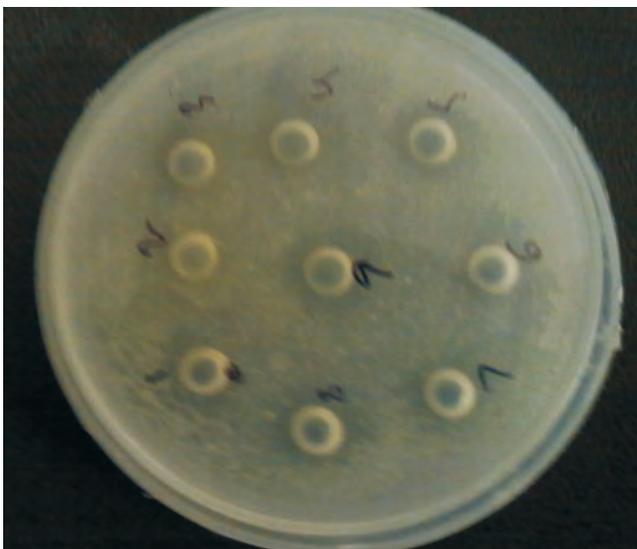
## 2.8. SDS–PAGE analysis

Partially purified enzyme from chitin exposed and non-exposed *V. cholera* reconfirmed via SDSPAGE analysis. A 12% polyacrylamide gel was prepared and partially purified enzyme from both cases (chitin exposed and non-exposed) was loaded in polyacrylamide matrix with standard protein ladder. Gel run for 3 h and after completion stained with commasee brilliant blur R250 and destained with excess of methanol in destaining buffer.

## 3. Results

### 3.1. Chitinase activity

Chitinase activity of both strains of *V. cholera* pathogenic and non-pathogenic accessed by well diffusion method. Colloidal chitin containing agar plates have incubated for 24 h with crude bacterial cultures with *E. coli* as control. All three bacterial strains that have used in current study have grown in different pH and without exposure of chitin, the results of Chitinase activity of *V. cholera* in different pH with *E. coli* as control on agar plate containing colloidal chitin is shown in Figure 1.



**Figure 1.** Chitinase activity of *V. cholera* in different pH with *E. coli* as control on agar plate containing colloidal chitin. In well 1, 2 & 3 *E. coli* grown at pH 6, 7 & 8 while in well 4, 5 & 6 *Vibrio cholera* WO5 grown at pH 6, 7 & 8 and in well 7, 8 & 9 *Vibrio cholera* VC 20 grown at pH 6, 7 & 8 in Luria broth.

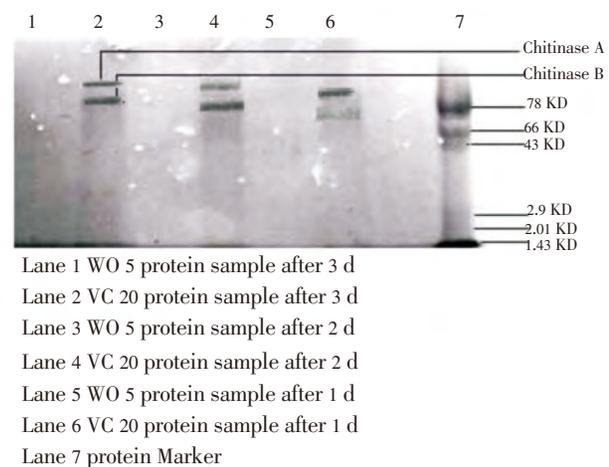
### 3.2. Time depended chitinase activity

To evaluate differential expression of chitinase gene under variant environment especially pH among both strain *Vibrio* and *E. coli* a time depended chitinase activity analysis had performed. Here all three strains were grouped in two class one chitin exposed and non-exposed prior to grow in different pH (Figure 2). All these strains have grown in prescribed condition for overnight at 37 °C. Colloidal chitin containing agar plates have used for chitinase assay by well diffusion method. From each class of bacterial culture, 25 µL of overnight grown culture has poured well in chitin agar plate. Further, plates have grown at 37 °C for 24, 48 and 72 h. Zone of inhibitions have measured and experiment

performed in triplicate to maintain reproducibility of results. Triplicate results (zone of inhibition) plotted against period of incubation and bacterial strain under chitin exposure and non-exposure.

### 3.3. SDS–PAGE analysis

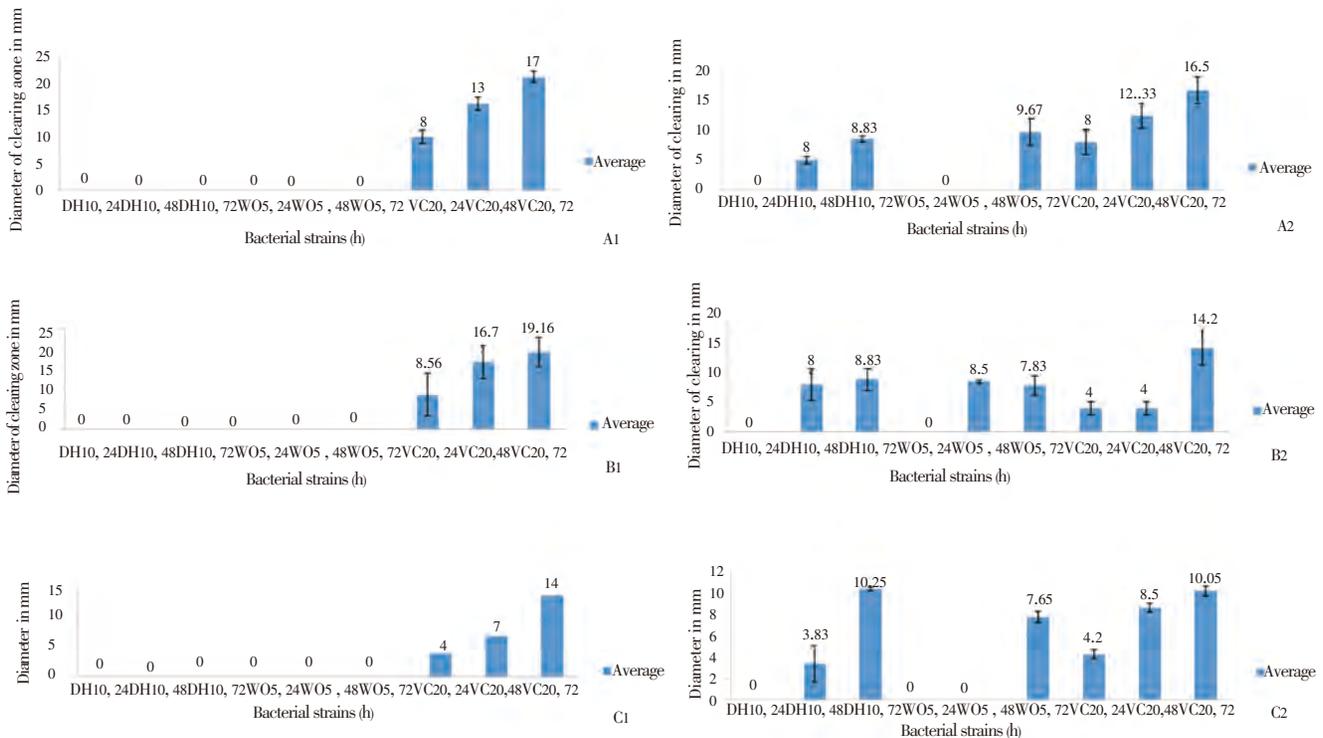
Based on the outcomes from time depended chitinase activity among all the bacterial strains used in the current study, we further moved to SDSPAGE (Figure 3). The purified chitinase from *V. cholera* VC 20 O1 Ogawa and *V. cholera* WO5 O1 Inaba had analyzed by SDSPAGE. Different purified fractions among different pH were loaded on 12% polyacrylamide gel run against standard protein ladder. Here we have comparatively analyzed both the strains of *V. cholera* under chitin exposure and grown at pH8. The fractions purified by cascades of chromatographic techniques where crude has taken from bacterial culture grown for different periods. The results obtained from SDSPAGE have predicted; the pathogenic strain *V. cholera* VC–20 O1 Ogawa under chitin exposure expressed chitinase significantly, while nonpathogenic strain *V. cholera* WO–5 O1 Inaba does not. However, results found in time dependent chitinase activity analysis were reproduced in SDSPAGE analysis which confirm expression of chitinase gene under chitin exposure in presence of CT gene in case of pathogenic strain. While in case of nonpathogenic strain under identical environment, we did not found even minimum traces of band in SDSPAGE[18].



**Figure 3.** SDS–PAGE analysis of purified chitinase in pathogenic and nonpathogenic strain of *V. cholera*.

### 3.4. Chitinase assay

Chitin exposed pathogenic and nonpathogenic strains of *V. cholera* subjected to colorimetric based chitinase assay. Chitin is one of most abounded carbohydrate in nature that comprise of long chain of *N*-acetylglucosamine. Chitin is one of prime source of carbon for many microbes including *Vibrio*. Genome of *Vibrio* enriched with gene encodes chitinase an enzyme convert polymeric form of chitin into available carbohydrate for microbe. Expression of chitinase gene is under control of various external stimuli like pH, temperature. To understand differential expression of chitinase gene two wild type strain of *Vibrio* VC 2001 (CT+) and WO 5 O1 (CT-) were grown in identical environmental

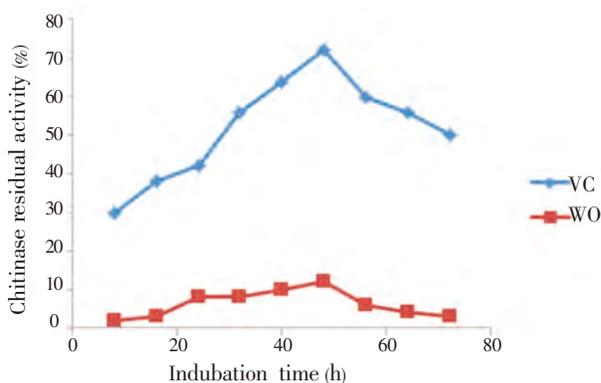


**Figure 2.** Time depended chitinase activity.

A1 B1 and C1 under chitin exposure while A2, B2 and C 2 without chitin exposure among pathogenic and nonpathogenic strain of *V. cholera* with *E. coli* DH10 as control.

conditions under exposure of chitin[19].

The chitin under enzymatic conversion in presence of chitinase produces of *N*-acetylglucosamine that reacts with p-dimethylaminobenzaldehyde which estimated by calorimetric analysis. The intensity of color led proportionate amount of release of *N*-acetylglucosamine by enzymatic conversion of chitin. The expression of chitinase gene under variant external factor like pH and presence of CT gene is prime concern of current study and we have found interesting results. Both the strains of *V. cholera* had grown in identical habitat under exposure of chitin (Figure 4). The growing cultures used for chitinase assay in different time interval in both the strain of *V. cholera*[20–22].



**Figure 4.** Chitinase activity evaluation under exposure of strain. Pathogenic and non-pathogenic *V. cholera* by colorimetric analysis.

#### 4. Discussion

The life threatening diarrheal consequences are still major challenges in concern with human health across the world and especially developing country where hygiene and sanitation is not optimal. Past few decades, in Asia and Africa many outbreaks have been reported leading to massive mortality[23–25]. Its *V. cholera* genomic plasticity tunes microbe to utilize habitat and surrounding resources for its growth and development. Among the 200 serogroups, only few are the pathogenic and led 70% of diarrheal problems. The serogroups O1 that is majority involves in diarrheal condition and newly O139, more refined and resistant to chemicals (drugs)[26,27]. The Newly emerging strains of *Vibrio* have adapted themselves to survive in changed environmental habitat and in the presence of antibiotics often used for medications. The in the current study we have evaluate chitinase activity of two wild type *V. cholera* in identical growth conditions. The *V. cholera* strains used in the current study having one difference VC20 O1 (pathogenic) naturally contains. CT gene while WO O5 O1 (non-pathogenic) lacks naturally. With the variant environmental conditions, CT gene also modulates expression of chitinase gene. Results have shown under the exposure of chitin in identical growing media both *Vibrio* responds differently, CT+ strain express chitinase gene significantly while CT- almost negligible. In the previous studies role of climatic conditions and CT gene was discussed separately among the different *Vibrio* strains[28]. Here we have found that CT gene is prime regulator of chitinase gene expression in pathogenic strain of *Vibrio*

with external habitat factors like pH and temperature. The regulated expression of chitinase gene in presence of CT gene is due to location of both genes; in same chromosome (chromosome-1) under same regulatory sequence<sup>[29]</sup>. Results from chitinase assay and time depended chitinase activity analysis have shown environmental conditions are the minor regulators while CT gene major ones. Further, SDS-PAGE analysis after partial purification of Chi-A and Chi-B proteins confirmed differential and controlled expression of chitinase in both the strain of *Vibrio*. The adaptation of *Vibrio* for utilization of chitin not just only for carbon source but it also provides ideal support for adherence that is essential for biofilm formation. Enzymatic conversion of chitin produces enormous monomers, *N*-acetylglucosamine which facilitates binding of pathogen to support. The chitin exposure also provides resistance toward harsh conditions like pH, essential while infection<sup>[30]</sup>. However, during *Vibrio* infection often we consume both pathogenic and non-pathogenic strains. The gastric pH cause death of majority of infected pathogen, while very small percent of infected *Vibrio* survives and led to diarrheal consequences. Despite this, concurrent chitin exposure to pathogen led thermal and chemical resistance among all the O1 and O139 serotypes<sup>[31]</sup>.

In the current study, we tried to expel differential behavior of pathogenic and non-pathogenic strains of *Vibrio* in variant habitats. As chitin is major carbohydrate in nature and majority of microbes utilize chitin as carbon source. However, *V. cholera* behaves differently under exposure of chitin, it's not only carbon source for their growth. Additionally it provides adaptation to pathogen to survive in harsh condition with expression of various toxins<sup>[32]</sup>. The current study becomes even more significant after evaluation of differential behaviors of pathogenic *Vibrio* in understanding spreading of *Vibrio* globally. Interestingly, many developed nations which have eliminated *Vibrio* in last few decades have suffered with cholera outbreaks. The migration of *Vibrio* in such places often happens due to the sea food transportation. Sea foods consumption has grown tremendously in last one decade as enriched in nutrients. As sea food consist of massive amount of chitin which provide ideal platform for *Vibrio* to grown even deep freezing<sup>[33,34]</sup>. From the previous investigation, repeated deep freezing cause expression of cold-shock proteins that favors and tunes microbial adaptations.

Among all the water-born diarrheal disease *V. cholera* driven cholera is one of them causing mortality world-wide. The current prospects of research are more focus to minimize outbreaks of cholera with finding new treatment regimes. The molecular mechanism of CT and other virulence factors and their expression in time depended and in variant habitat is prime research focus many researcher. Here, we have investigated the role and interaction of CT gene in variant expression of chitinase gene in genetically identical *V. cholera* with one difference of CT gene<sup>[35]</sup>. The emerging new resistance strains of *Vibrio* are major threats for conventional medications and global spread of pathogen. The sea foods, enriched nutritional foods contaminated with pathogenic *Vibrio* are prime concern for molecular research to understand complete etiology of pathogen in order to minimize negative consequences<sup>[36]</sup>.

The differential behavior of pathogen against external and internal factor not only provides natural adaptation for its survival but also emergence of new potent sub-species. Molecular talk between CT gene and chitinase gene

allow pathogen to adhere at specific time in specific place. Interaction of CT gene with chitinase open new arena to understand etiology of accessory proteins plays significant role *Vibrio* life cycle form infection to pathogenesis. Chitin is one of them, which allow pathogen to survive in harsh conditions with maintained or improved pathogenesis. The current study concludes, management of *Vibrio* driven disease does not accomplished with medication or newer generations drugs, precisely it requires finding of molecular refinements running in pathogen leading to new and potent strains.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgements

We would like to thank Management and Principal of R.V.R. & J.C. College of Engineering for providing laboratory facility for smooth experimental work. I would like thank our laboratory technician while supporting proper use and recommended disposal of pathogenic strain of candidate organism. I am thankful to Dr. Ramamurthy, Scientist, National Institute of Cholera and Enteric Disease (NICED) Kolkata, West Bengal, India for providing bacterial strains of *V. cholera*.

### Comments

#### Background

Virulence of a pathogen depends upon factors derived from mechanisms of adaptation to its environment. In the present study, an attempt has been made to understand the microenvironmental factors that influence the pathogenicity of the two strains of *V. cholera*.

#### Research frontiers

Selection of a useful model for understanding the interactions of a pathogen with food and other components of its environment and accordingly adapt to the host and express virulence.

#### Related reports

Well diffusion method for chitinase activity test, chitinase assay and colorimetric method employed in the study are standard methods used by researchers world wide.

#### Innovations & breakthroughs

Modulation of expression of chitinase gene by CT gene is an interesting finding, strongly suggesting the need for thorough investigation into molecular talk that favours control of the disease along with novel drug formulations to combat infections.

#### Applications

Prevention, control and treatment of the epidemic cholera in humans and other animals.

#### Peer review

Quite a good effort made and further studies to be

continued for coming up with a comprehensive approach to combat the disease.

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