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Cytotoxic isolates of *Helicobacter pylori* from Peptic Ulcer Diseases decrease K⁺-dependent ATPase Activity in HeLa cells

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Abstract

Background: *Helicobacter pylori* is a Gram negative bacterium that plays a central role in the etiology of chronic gastritis and peptic ulcer diseases. However, not all *H. pylori* positive cases develop advanced disease. This discriminatory behavior has been attributed to the difference in virulence of the bacteria. Among all virulence factors, cytotoxin released by *H. pylori* is the most important factor. In this work, we studied variation in *H. pylori* isolates from Indian dyspeptic patients on the basis of cytotoxin production and associated changes in K⁺-dependent ATPase (one of its targets) enzyme activity in HeLa cells.

Methods: The patients were retrospectively grouped on the basis of endoscopic and histopathological observation as having gastritis or peptic ulcer. The HeLa cells were incubated with the broth culture filtrates (BCFs) of *H. pylori* isolates from patients of both groups and observed for the cytopathic effects: morphological changes and viability. In addition, the K⁺-dependent ATPase activity was measured in HeLa cells extracts.

Results: The cytotoxin production was observed in 3/7 (gastritis) and 4/4 (peptic ulcer) *H. pylori* isolates. The BCFs of cytotoxin producing *H. pylori* strains reduced the ATPase activity of HeLa cells to 40% of that measured with non-cytotoxin producing *H. pylori* strains (1.33 μmole Pi/mg protein and 3.36 μmole Pi/mg protein, respectively, $p < 0.05$). The decreased activity of ATPase enzyme or the release of cytotoxin also correlated with the increased pathogenicity indices of the patients.

Conclusions: Our results suggest that the isolation of cytotoxic *H. pylori* is more common in severe form of acid peptic diseases (peptic ulcer) than in gastritis patients from India. Also the cytotoxin released by *H. pylori* impairs the ion-transporting ATPase and is a measure of cytotoxicity.

Background

Helicobacter pylori is a spiral Gram negative, microaerophilic bacterial parasite that inhabitates gastric epithelium [1]. The long-term *H. pylori* infection has been found associated with gastritis, gastric and duodenal ulcer, B-cell lymphoma and adenocarcinoma of the stomach [2–4]. *H. pylori* infection is common in both developed and developing countries. It is estimated that in developed countries 30–50% of the adult population is infected. In developing countries, the prevalence of *H. pylori* infection is noted even higher-approximately 80% [5,6]. Interestingly, not all the *H. pylori* infected individuals develop

peptic ulcer or gastric cancer. A significant number of patients have milder form of disease, like inflammation in stomach and duodenum [7,8]. Such discriminatory behavior of *H. pylori* can be linked to the ability or inability of the bacteria to express various virulence factors. These virulence factors help *H. pylori* in adapting to its environment and are subject to variation from one geographical location to another [9–12]. The virulence factors of *H. pylori* responsible for its pathogenesis and survival in harsh acidic environment include spiral morphology, motility, lipases, protease and urease enzymes, and cytotoxin production [9–12].

The expression of cytotoxin-associated gene (cagA and/or vacA) and cytotoxin production by *H. pylori* strains has been studied and correlated with their enhanced pathogenic potential [13–15]. But no explicit cause-effect relationship has been demonstrated between the cytotoxin and the clinical outcome in the patients. Secondly, depending on the geographical area, the genotypic and phenotypic differences among *H. pylori* strains exclude generalization of studies done in developed countries. These differences arise, among other factors, from eating habits, economic status and prevailing hygienic conditions [16–18]. In our previous study, we investigated the association between the differences in zymograms of *H. pylori* isolates and clinical presentation of the disease in Indian dyspeptic patients [19]. Over 40% of the *H. pylori* isolates were found belonging to a biotype that expressed alkaline phosphatase, esterase-lipase, naphthol-AS-beta-1-phosphohydrolase, esterase and acid phosphatase [19]. However, no correlation was found between the biotype and disease status of the patients. Other investigators also failed to find any association between the cytotoxin gene/antigen in *H. pylori* isolates from Indian patients with the clinical outcome [20,21]. To further investigate the strain-differences among *H. pylori*, in the present study, the isolates were studied for cytotoxin production and the changes in ATPase activity of target (HeLa) cells. Since amino terminal sequence of this toxin shows partial homogeneity with the sequences of ion-transporting ATPase, it has been suggested that ATPase may be the one of the targets of cytotoxin produced by *H. pylori* [13].

In the present study, we found that the cytotoxic *H. pylori* were isolated commonly from peptic ulcer patients. And only half of *H. pylori* isolates from, gastritis, the milder form than ulcer, were cytotoxic. In addition, the cytotoxic *H. pylori* strains decrease the ATPase activity of HeLa cells, which correlates with the severe damage and increased pathogenicity indices of the patients.

Methods

Patients and disease status

A total of 11 isolates of *H. pylori*, isolated from antral biopsies of patients with gastritis (Group I, n = 7) and peptic ulcer (Group II, n = 4) were included in the present study. These patients (male 9, female 2) ranged in age from 10–38 years (mean 30.2 years) and none of the patients received any antimicrobial therapy for at least four weeks prior to the study. The patients showed typical clinical symptoms of peptic ulcer diseases: dyspepsia, heartburn and epigastric pain. They underwent gastroduodenoscopy for both visual examination and biopsy collection. The disease status of the patients was defined on the basis of endoscopic and/or histopathological positivity for inflammation.

At least four antral gastric biopsies were obtained from each patient using gastrovideoendoscope (Olympus, Japan) for histopathology, rapid urease test, modified Gram's staining and culture of *H. pylori*. For histopathology, the biopsy was collected in 10% formalin buffered-saline. Tissue sample was dehyrated, infiltrated, embedded in paraffin wax, sectioned and stained with Haematoxylin and Eosin and Giemsa stain. The slides were then examined for histopathological changes [19]. In addition the biopsies were examined for *H. pylori* by rapid urease test and microscopic examination of impression and crushed smear after staining with modified Gram's stain as per the method described earlier [19].

Reference cytotoxin producing strain, *H. pylori* CCUG 17874 was obtained from Culture Collection University of Goteborg, Goteborg, Sweden.

Electron Microscopy

Transmission electron microscopy of the biopsies was performed as described earlier [22,23]. Briefly, the biopsies were fixed in 3% glutaraldehyde-2% paraformaldehyde solution (3 h) followed by fixing in 1% osmium teraoxide (4 °C for 1 h) in 0.1 M cacodylate buffer. The samples were dehydrated in diluted aqueous acetone, infiltrated and embedded in plastic mixture. Finally, the samples were trimmed into ultrathin sections (1 μm) using Ultratome 8800 (LKB, USA). The sections were stained with uranyl acetate and lead citrate solutions before examining under transmission electron microscope (Phillips EM 416LS, Holland) at an accelerating voltage of 80 KV. Few representative areas were photographed on orthochromatic electron microscopic sheet films (Kodak, UK) under different magnifications. For scanning electron microscopy of *H. pylori*, the bacteria were fixed using 0.1% poly-L-lysine and dehydrated in ethanol and ethanol: isoamyl acetate solution. The samples were dried in Balzer's union critical point dryer by gradual replacement of isoamyl acetate with liquid CO_2. The dried samples were mounted on

aluminium stubs, coated with Au-Pd alloy (160 Å) and scanned (Scanning electron microscope, Phillips 515, Holland) at varying magnifications [24].

Isolation of H. pylori *and preparation of broth culture filtrates (BCFs)*

The endoscopic biopsies were cultured on Columbia Chocolate agar plates (Difco, USA) containing 7% defibrinated sheep blood and antibiotics (6 mg/ml vancomycin, 1000 U/ml polymyxin B and 2 mg/ml amphotericin B; Sigma, USA) to inhibit the growth of commensals. Bacterial colonies were grown under microaerobic condition at 37°C for 48–72 h. Finally, translucent *H. pylori* colonies were identified on the basis of spiral morphology and presence of oxidase, catalase and urease enzyme activity as per the method described earlier [19].

The H. pylori isolates thus cultured were grown into 10 ml of Columbia liquid broth (Difco, USA) containing 1% haemin, 1% starch and antibiotics under microaerobic conditions [25]. The BCFs were prepared by removing bacteria after centrifugation at 8,000 × g for 15 min followed by filtration through 0.22 μm filters (Millipore, USA) as described earlier [26].

Cytotoxicity assay

Human cervical epithelial cells (HeLa cells) were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in Eagle's minimum essential medium (MEM, Nissui, Japan) supplemented with 10% fetal bovine serum (Gibco, USA) and 50 μg/ml gentamicin (Sigma, USA) in 12 well flat bottom plates (Nunc, Denmark) to produce semiconfluent monolayer at 37°C in a humidified atmosphere of 5% CO_2. After washing the cells thrice with serum-free MEM, the cells were incubated with diluted BCFs (1:10 in MEM) of *H. pylori* isolates and the reference *H. pylori* CCUG 17874. The incubation was continued for additional 24–48 h under same conditions. Un-inoculated Columbia broth filtrate (passed through 0.22 μm filter) was used as negative control. The cells were observed for morphological changes such as vacuolization and rounding of cells using inverted microscope (Nikon, Japan). The viability of HeLa cells was determined as per trypan blue dye exclusion method.

K^+-dependent Adenosine triphosphatase (ATPase) activity

After 24 h incubation with *H. pylori* BCFs or negative control, the HeLa Cells were washed thrice with ice-cold 0.15 M NaCl and were scraped in 0.1 M Tris buffer solution (pH 7.4). The cells suspension was bath-sonicated for 15 sec in ice-cold water. Measurement of K^+-dependent ATPase activity was carried out as per the method of Abdel Latif etal [27]. Briefly, the cell homogenates were incubated in an assay buffer containing 4.5 mM ATP, 100 mM NaCl, 20 mM KCl and 0.1 M Tris HCl (pH 7.4) and 0.5 mM ATP for 20 min at 37°C. The reaction was stopped by adding 100 μl of 50% trichloroacetic acid and the reaction mixture was centrifuged at 1500 × g for 10 min. Release of inorganic phosphate (Pi) was assayed by incubating the supernatants with 2.5 N H_2SO_4, 5% ammonium molybdate and equal amount of reducing agent (0.1% 1-amino 2-naphthol 4-sulphonic acid, 19.5% sodium metabisulphite and 0.38% sodium sulphite solution) at room temperature for 10 min [28]. Optical density was read spectrophotometrically at 660 nm. Potassium dihydrogen phosphate solution (1.4 mg/ml) in deionized water was used as standard for measurement of Pi release. To normalize the Pi release, total protein content of HeLa cell homogenates was estimated by Lowry's method [29]. Specific ATPase activity was calculated in terms of μmole Pi liberated per mg protein. All the assays were carried out in triplicate.

Statistical Analysis

Data were analyzed statistically using Analysis of Variance (ANOVA) and Chi-square test by Macintosh Statview software. The p value less than 0.05 was considered significant.

Results

This study was done to investigate the degree of gastric damage in relation to the ability of *H. pylori* to secrete cytotoxin in patients with peptic ulcer diseases. All patients complained of dyspepsia, heartburn and epigastric pain for a period not less than 1 year. The presence of *H. pylori* in antral biopsies was established by rapid urease test, modified Gram's staining of smear and culture of the biopsies. The gastric and duodenal damage was assessed by gastro-duodenal endoscopy and histopathology of Haematoxylin & Eosin stained biopsy samples by light microscopy. The details of the damage were ascertained at cellular level by electron microscopy. Cytotoxin production was determined in BCFs of *H. pylori* isolates from biopsies on HeLa cells. To further investigate the cytotoxicity, effect of cytotoxin on activity of K^+-dependent ATPase in HeLa cells was studied. A pathological index was calculated for each patient as follows:

Pathological index = Endoscopy* + Histopathology** + Cytotoxicity on HeLa cells ***.

*Endoscopy: Normal = 0, Gastritis = 1, Ulcer = 2

**Histopathology: Normal = 0, Chronic superficial gastritis = 1

***Cytotoxicity on HeLa cells: None = 0, Rounding and vacuolization of HeLa cells = 1.

Table 1: Endoscopic and histopathological details of patients.

	Endoscopic findings	Histopathological findings
Group I:		
1	Antral gastritis	Chronic superficial gastritis
2	Antral gastritis	Chronic superficial gastritis
3	Normal	Chronic superficial gastritis
4	Normal	Chronic superficial gastritis
5	Normal	Chronic superficial gastritis
6	Antral gastritis	Chronic superficial gastritis
7	Antral gastritis	Chronic superficial gastritis
Group II:		
1	Duodenal ulcer	Chronic superficial gastritis
2	Gastric ulcer	Chronic superficial gastritis
3	Duodenal ulcer	Chronic superficial gastritis
4	Duodenal ulcer	Chronic superficial gastritis

Identification numbers given to the patients are arbitrary numbers.

All the patients (10–38 years of age) included in the study, presented with common dyspeptic symptoms such as nausea, vomiting and epigastric pain. With the use of endoscope, signs of hyperemia, hemorrhage, friability, erosions and erythema were observed in the stomach of patients having antral gastritis (Group I). Two patients (Group I) showed no sign of inflammation in stomach or duodenum after endoscopic examination (Table 1). In Group II patients, gastric and duodenal ulcers were observed in the form of white patches in stomach and different parts of the duodenum. Chronic superficial gastritis was diagnosed in all the patients (Group I and II) and was recognized histologically by the flattening of microvilli, presence of inter-epithelial and interstitial polymorphs infiltration in addition to the lymphocytes and plasma cells in lamina propria. Epithelial degeneration and regeneration were also evident with and without any loss of glands.

The rapid urease test on gastric biopsies from these patients was positive indicating the presence of *H. pylori*. Light microscopy of biopsy smears, stained with modified Gram's stain, showed spiral shaped *H. pylori* in groups that were unevenly distributed in the biopsies. Smear examination was taken as positive when *H. pylori* was demonstrated in at least one of the impression or crushed biopsy preparations. Comma and donut forms of *H. pylori* were also occasionally seen in primary biopsy smears. The histopathological findings and colonization of *H. pylori* were confirmed further by transmission electron microscopy. The electron micrographs of the sections of antral biopsies of the patients and *H. pylori* isolates are shown in figure 1. Electron microscopy confirmed the histopathological findings at cellular level. Infiltration of inflammatory cells, degeneration of epithelial layer, flattening of microvilli and *H. pylori* were clearly visible (Fig. 1b and 1c). The intracellular vacuolization was also visible in *H. pylori* infected gastric biopsy (Fig. 1b). The bacteria were visible in close contact with gastric epithelium and were sometimes seen residing at the intercellular junctions of the gastric epithelial cells layer (Fig. 1c). At the site of adhesion of *H. pylori*, a cup like invagination was observed on the surface of gastric epithelial cells (Fig. 1d). Scanning electron microscopy of all the samples confirmed the spiral shape and smooth, curved surface of bacteria. Representative micrographs are shown in figure 1. In addition, the bacteria were found to possess flagella (figure not shown). We could not see any of the fine fimbrial or fibrillar structures on the surface of any of the *H. pylori* isolates by scanning electron microscopy, however, very hazy structures were visible in transmission electron micrographs of the biopsies at the site of attachment of *H. pylori* to the epithelial cell (Fig. 1d).

Once biopsies were cultured under microaerobic conditions, the translucent colonies of bacteria were identified by testing for the presence of various enzymes unique to *H. pylori*. All the isolates were positive for catalase, oxidase and urease enzymes. Even after further subcultures on Columbia chocolate agar plates and in liquid broth medium, *H. pylori* remained positive for these enzymes and showed spiral morphology. None of the isolates, however, showed cocci form of *H. pylori* in primary as well as in subsequent cultures.

When BCFs of *H. pylori* from ulcer patients were added to the HeLa cells, cytopathic effects were observed marked with rounding of cells and vacuolization (Fig. 2). Reference strain *H. pylori* CCUG 17874 also showed similar cytopathic effects, i.e., cell rounding and vacuolization. While all the isolates from ulcer patients were cytopathic, only 3/7 of *H. pylori* isolates from Group I patients showed such effects on HeLa cells. The electron micrographs of gastric biopsies from cytotoxin producing *H. pylori* infected patients with peptic ulcer also showed vacuolar structures, similar to those observed *in vitro* in HeLa cells. These structures were localized on the epithelial surface at the site of attachment of *H. pylori* (Fig. 1b). No attempt was made to quantify the degree of cytotoxicity as seen by microscope except qualitative designation. The cells remained viable even after 48 h of treatment with BCFs.

One way to quantify the cytotoxicity of *H. pylori* was to measure the activity of K^+-dependent ATPase that had been shown sensitive to *H. pylori* cytotoxin [30]. The activity was expressed as μmole of Pi liberated per unit of protein. BCFs from cytotoxin producing *H. pylori* CCUG 17874 strain and clinical isolates from Group II (peptic ulcer) patients caused a significant decrease in K^+-depend-

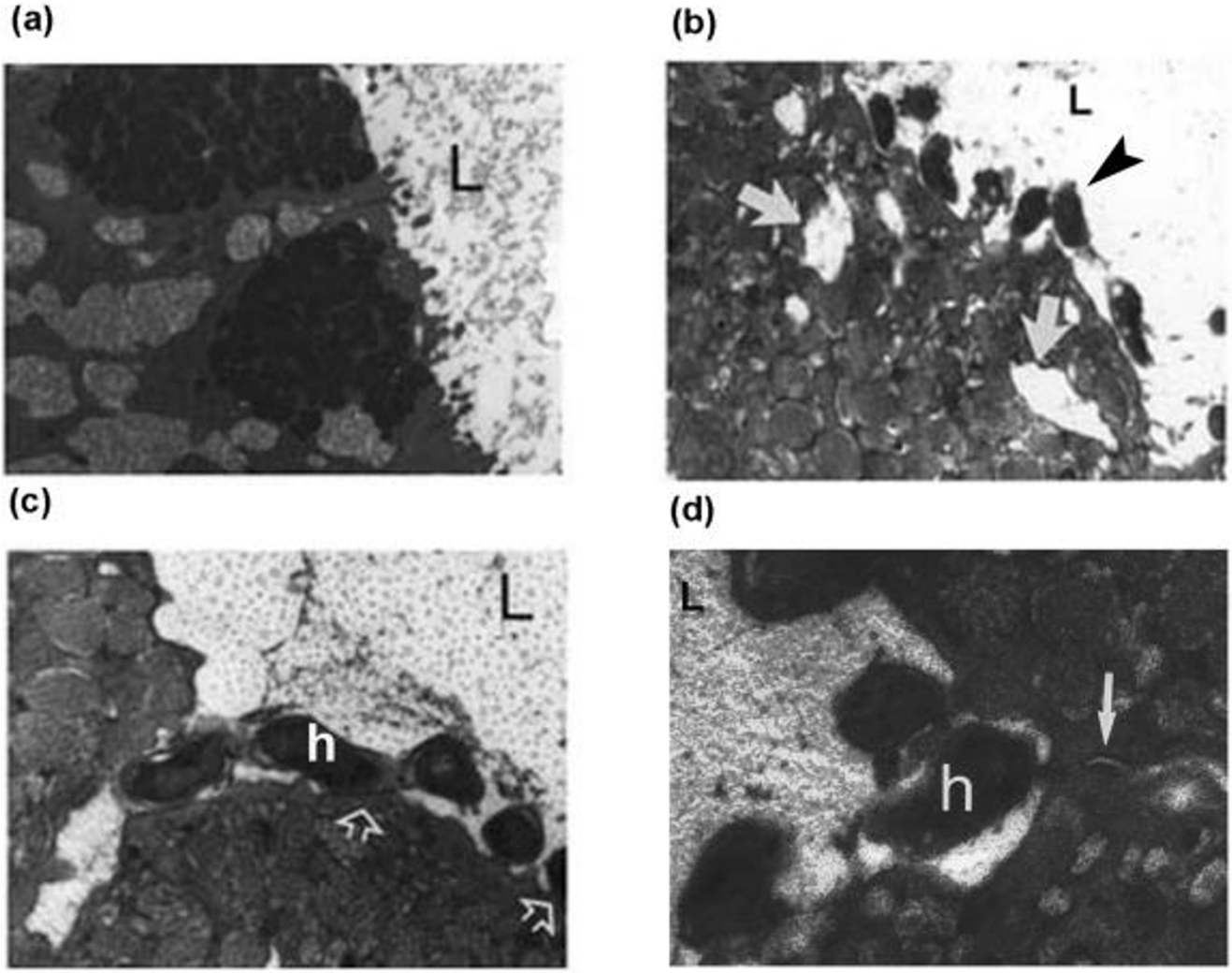

Figure 1
Electron micrographs: Transmission electron micrographs of antral biopsies of (a) *H. pylori* negative normal gastric epithelium, L-gastric lumen (× 4,400), (b) *H. pylori* (cytotoxin producing) infected gastritis patient showing vacuolar regions in the epithelium (white arrowhead), L-gastric lumen, (× 7,100), *H. pylori* (black arrowhead) (c) h-*H. pylori* on the apical and intercellular junctions of epithelial layer (arrowhead), L-gastric lumen, (× 10,400), (d) cup like invaginations on the surface of gastric epithelium at intercellular junction (arrowhead), h-*H. pylori*, L-gastric lumen (× 21,000).

ent ATPase activity of HeLa cells (1.01 μmole/mg protein) as compared to control, i.e., medium only (2.6 μmole/mg protein, $p < 0.05$) (Fig. 3). Heat inactivation (100°C for 15 min) of BCFs of these isolates abolished the cytotoxicity and the ATPase activity was found comparable to controls. Thus, a heat labile factor was responsible for cytotoxicity. On the other hand, the mean ATPase activity of HeLa cells incubated with BCFs of *H. pylori* isolates (n = 6, ATPase activity was not measured in one of the four non-cytotoxic isolates from Group I) from Group I patients did not show any change form the medium control (Fig. 3). When the group I was divided into two subgroups, according to the microscopic cytopathy on HeLa cells as described above, the same 3/6 cytopathic isolates decreased ATPase activity of HeLa cells (1.76 μmole/mg protein, $p < 0.05$ versus medium); other 3/6 non-cytotoxic isolates had no statistically significant effect on ATPase activity of HeLa cells (3.36 μmole/mg protein). A correlation was observed between the *H. pylori*-induced reduction in HeLa cell ATPase activity and the pathological indices calculated for the patients ($p = 0.005$, Chi-square and ANOVA). All six patients with pathological

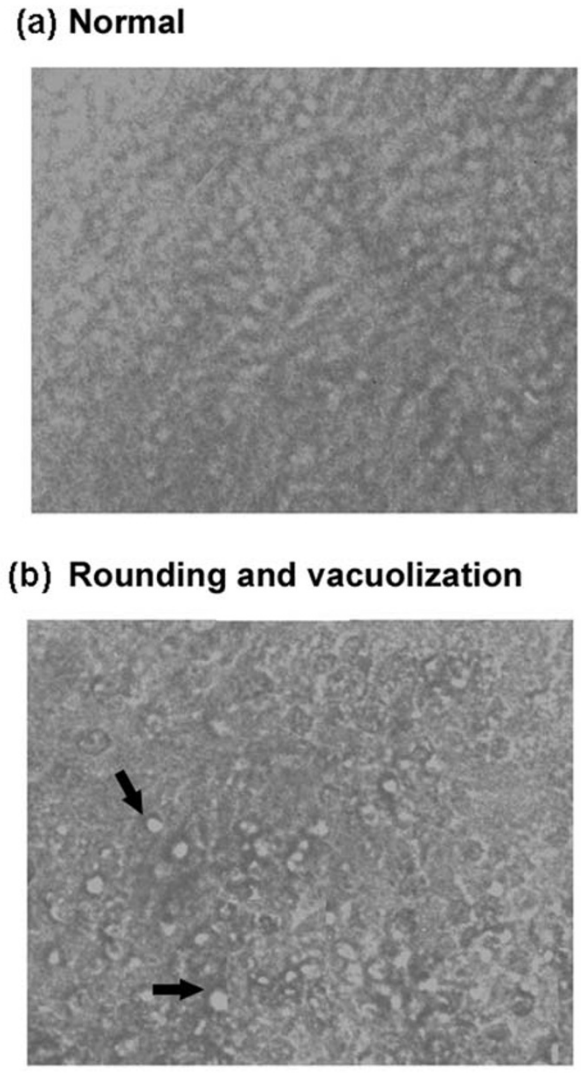

Figure 2
Effect of BCFs of cytotoxic *H. pylori* on morphology of HeLa cells. HeLa cells incubated with (a) medium alone (normal morphology) and (b) BCF of cytotoxic *H. pylori*. Rounding and vacuolization of cells has been shown (arrowhead). The photomicrographs of HeLa cells, shown here were taken after 24 h of incubation with BCF or medium from one representative experiment.

index >2 yielded *H. pylori* isolates that reduced HeLa cell ATPase activity to 1.32 μmole/mg protein (2.6 μmole/mg protein control activity). Chi-square distribution for this correlation is shown in figure 4.

Discussion

Helicobacter pylori colonizes gastric epithelium and the presence of *H. pylori* infection is one of the major determinants in the development of peptic ulcer diseases. The clinical outcome of *H. pylori* infection is multifaceted because only few of the infected individuals develop the most severe peptic ulcer or gastric carcinoma [31]. Previous studies showed that biologically active cytotoxin (VacA) is released by certain *H. pylori* strains that possess both cagA (cytotoxin-associated gene) and vacA gene [32,33]. On the basis of cagA gene, attempts have been made to perform genotyping of *H. pylori* using cagA (pathogenicity island) or iceA (induced by contact with epithelium) gene probes [34,35]. The aim of these studies was to identify the virulent strains and their correlation with the disease outcome, but no consensus on the association of cytotoxin release or expression of cagA gene or cagA antigen with the disease status has been obtained [36,37]. In fact, extreme diversity was found in the prevalence of cytotoxin producing *H. pylori* strains in populations of different geographical regions [38–41].

There have been very few studies available on the characteristics of *H. pylori* strains from India, where the infection rate is very high-over 80% [42]. Presence of cagA gene or antibodies to cagA gene was not found associated with the outcome of the peptic ulcer disease in Indian patients [20,21,43,44]. Also, in our previous study we could not find any association between various biotypes of *H. pylori* (on the basis of zymogram and antibiogram) with the disease outcome [19]. In this context, the variation in the degree of cytotoxicity or degree of damage induced by cytotoxic *H. pylori* strains has also not been studied. Here, we investigated the relationship between cytotoxin producing *H. pylori* strains and the ensuing gastric mucosal damage in a small number of patients. Although the role of host response in mounting an innate and adaptive immune response cannot be ruled out in the outcome and severity of disease; we focused only on the virulence potential of *H. pylori*. Thus, *H. pylori* was isolated from antral biopsies and after characterization, the bacteria was studied for cytotoxicity in HeLa cells.

Previously it has been reported that the spiral *H. pylori* changes its shape to cocci form after isolation and storage and looses its potent virulence properties [45]. However, we found that all the isolates, included in this study, were spiral shaped and were homogenous in their morphological and biochemical characteristics as evident from electron microscopy and the activity of urease, catalse and oxidase enzymes. The only variation was in their ability to produce cytotoxin as was evident from the cytopathic effects on HeLa cells. All the isolates from peptic ulcer patients produced cytotoxin and only 3/7 isolates from patients of gastritis group were cytotoxic. Following the

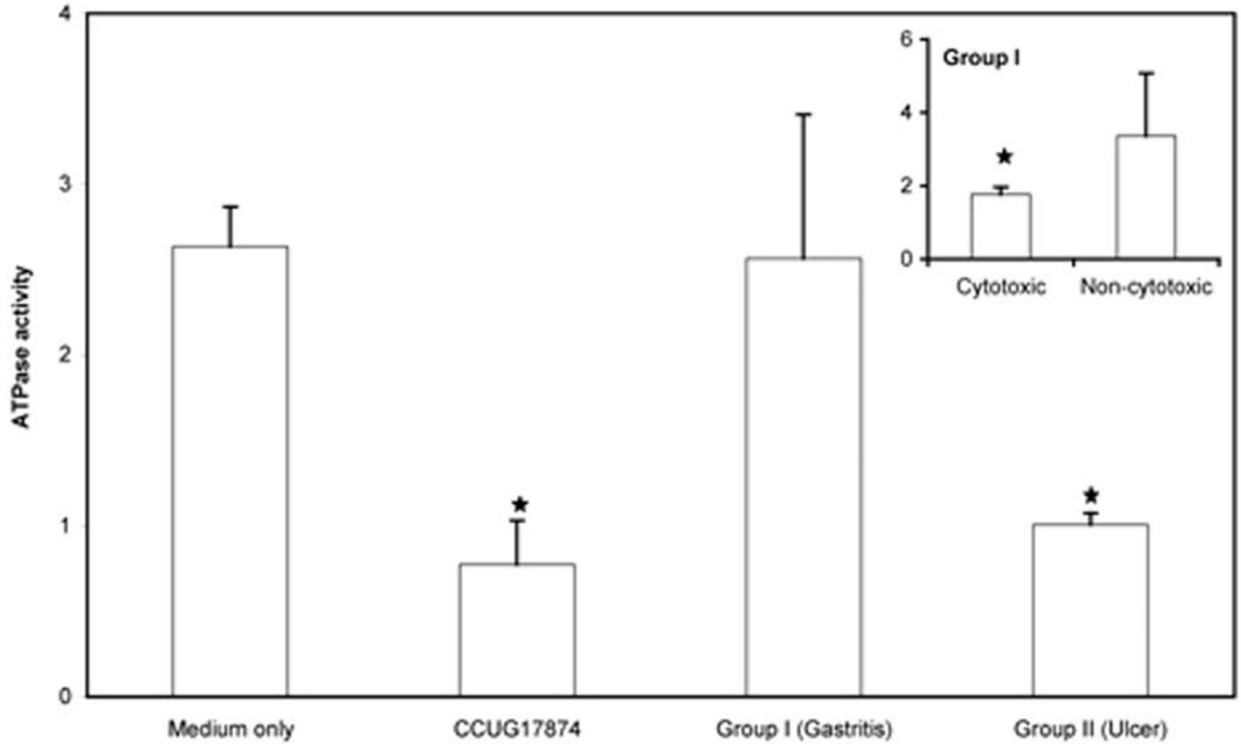

Figure 3
Effect of *H. pylori* BCFs on K^+-dependent ATPase activity of HeLa cells. ATPase activity (μmole Pi released/mg protein) of HeLa cells was measured after incubating the cells with BCFs of *H. pylori* isolates. Values are shown as mean (SEM) of three independent measurements. *$p < 0.05$ as compared to medium control.

endoscopy, these patients were treated with anti-*H. pylori* drug regimen. This is quite possible that if gastritis patients infected with cytotoxic *H. pylori* strains were left untreated, they would have eventually developed peptic ulcer. However, the patients were not followed further.

To further quantitate the degree of damage induced by *H. pylori* cytotoxin, we measured K^+-ATPase activity of HeLa cells. Eukaryotic cells are known to express two types of ATPase enzyme-V-type (H^+-ATPase) and P-type ATPase (Na^+K^+-ATPase). *H. pylori* cytotoxin targets K^+-dependent ATPase and impairs its activity [46]. Earlier, cytotoxin of *H pylori* CCUG 17874 has been shown to decrease the ATPase activity of MKN28 cells [30]. However, no correlation was made with the clinical outcome. Here, we found that both cytotoxic *H. pylori* isolates from Group I (histological gastritis) and Group II (gastric and duodenal ulcer) patients inhibited ATPase activity of HeLa cells. In contrast, non-cytotoxic isolates from Group I (gastritis) patients effected no change in ATPase activity of HeLa cells homogenates. There was a strong correspondence between reduced ATPase activity of HeLa cells and cytotoxin production by *H. pylori*. This effect is not due to ammonia released by the action of *H. pylori* urease, as all the strains, regardless of source, were positive for urease activity. However, presence of any other toxic substance(s) (like proteases) besides cytotoxin in BCFs cannot be ruled out. Earlier, several clinical and *in vitro* studies from isolated parietal cells suggested that *H. pylori* infection inhibits acid secretion [47,48] and gastric H^+-K^+-ATPase alpha-subunit gene expression [49]. Our data demonstrate here that the secreted cytotoxin component(s) of *H. pylori* is responsible for the inhibition of ATPase activity of epithelial cells.

Conclusions

Our study suggests that cytotoxin component of *H. pylori* acts on its biochemical target: ATPase in epithelial cells and inhibits the enzyme activity. Moreover, on the basis of isolation of cytotoxic *H. pylori* strains from ulcer patients, it can be speculated that cytotoxin causes severe damage to the gastric epithelium. Given the paucity of

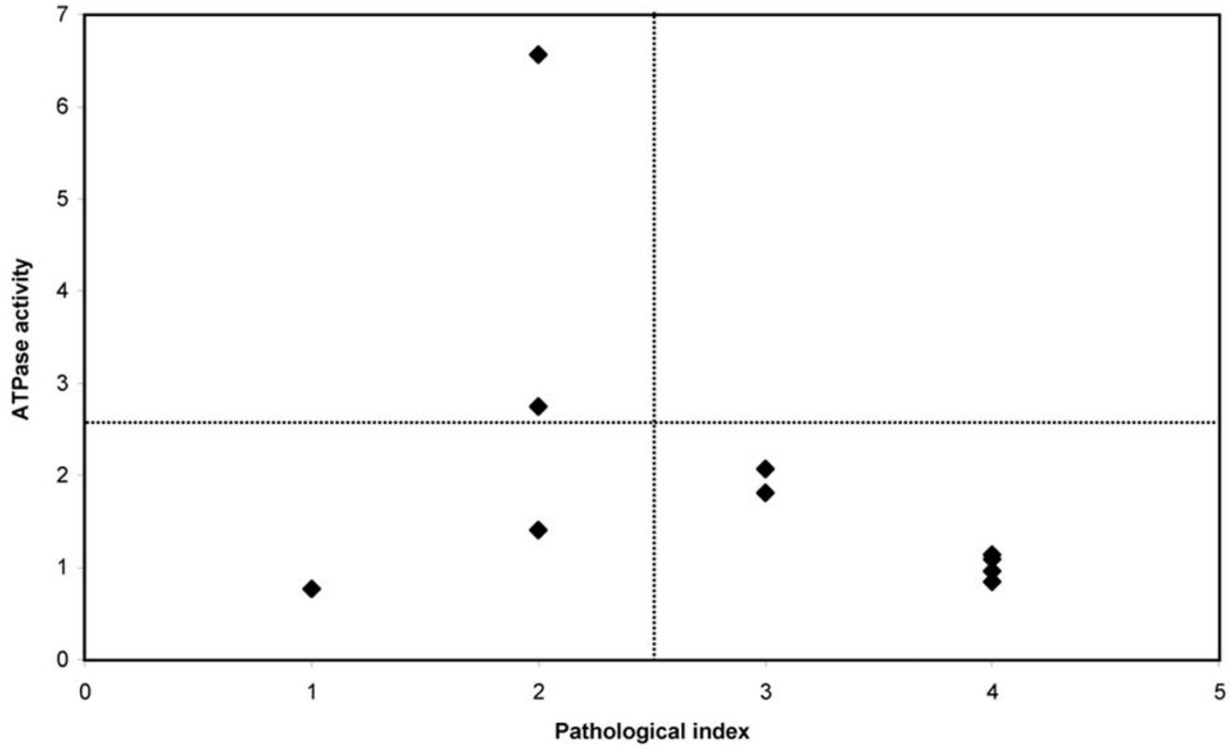

Figure 4
Scattergraph of K^+-dependent ATPase activity (μmole Pi released/mg protein) of HeLa cells incubated with BCFs of *H. pylori* isolates from patients with different pathological indices.

information about virulence factors unique to geographical location outlined by India, this study adds to the available knowledge base about *H. pylori*. In developing world where infections with antibiotic-resistant *H. pylori* is very common, the application of therapeutic or prophylactic vaccines can be useful in preventing *H. pylori* infection [50]. So far, vaccination studies have not been completely successful, the cytotoxin released by *H. pylori* is still at the first place on the list of many putative virulence factors and a potent candidate for vaccine production. However, more studies on the activity of cytotoxin at molecular level, host response in an animal model should provide the basis for the design of an effective vaccine.

Competing Interests
None declared.

Authors' Contributions
AA participated in designing and coordinating the study. SA performed the experiments, analyzed results and coordinated the study.

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