EFFECT OF STRESS ON PRODUCTION OF HEAT LABILE ENTEROTOXIN BY ESCHERICHIA COLI

*A Hegde, GK Bhat, S Mallya

Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is an important pathogen responsible for secretory diarrhoea especially in developing countries.[1-3] These bacteria produce a heat labile enterotoxin (LT) and heat stable enterotoxin (ST).[4] LT, involved in the pathogenesis of secretory diarrhoea caused by ETEC, is highly antigenic and resembles cholera toxin.[5] A number of toxicological as well as immunological tests for the demonstration of LT have been reported. These tests have depended either on animal, tissue culture facilities, radioactive or spectrophotometric registration making them unsuitable for use in less well equipped laboratories or in the field. A simple, reliable and practical assay is necessary for the detection of LT. The GMI ganglioside enzyme linked immunosorbant assay (GMI ELISA) is a sensitive and accurate immunological method for detection of LT-producing *E. coli*.[6]

The production of LT by ETEC is affected by several environmental conditions.[7] When ETEC infects a person it will have to survive in different stressful conditions such as varying temperature, variations in pH, osmotic stress and nutrient limitation. These stress factors may influence production of virulence factors by ETEC. There is not much data available on the effect of stress on LT production. The objectives of this study were to determine the effect of temperature, pH, osmotic stress and nutritional limitation on production of LT by ETEC by using in-house GMI-ELISA technique.

Materials and Methods

**Bacteria**

Four *E. coli* strains, consisting of one standard strain, *E. coli* MTCC 723 (Colonization factor antigen I and heat labile/heat stable enterotoxin producer obtained from Microbial Type Culture Collection [MTCC], Chandigarh, INDIA) and three clinical isolates that produced LT (detected by using GM1-ELISA) namely *E. coli* S001, S004, and S007 were used in the study. The clinical strains were isolated from faeces of children suffering from acute diarrhoea. The bacteria were preserved at minus 20°C in nutrient broth containing 20% glycerol as a cryopreservative. For daily use, the cultures were maintained on nutrient agar slope at 40°C, which were used for one month only. For further use, fresh nutrient agar slope cultures were made from the stock.

Preparation of the bacterial inoculum

Each bacterial strain was plated on tryptic soy agar containing 0.6% yeast extract and incubated at 37°C for 18 hours. The cells were washed, thrice, in sterile physiological saline (0.85% NaCl w/v) and finally suspended in saline to get a solution with OD$_{600}$ 0.1 (corresponds to approximately 1x10$^6$ cells /ml, confirmed by surface plating on nutrient agar). In this experiment Mundell's medium was used.[8] The medium was dispensed in 10 ml volume in 100
ml Erlenmeyer flasks. Bacterial suspension, prepared as described above, was used to inoculate the culture medium to achieve an initial concentration of approximately 1x10⁶ cells /ml in each flask.

Effect of temperature, pH, nutrient limitation and osmotic stress on LT production

The effect of temperature, pH, nutrient limitation and osmotic pressure on LT production was studied as described previously.[9,10] Bacteria was grown at 34°C, 37°C, 40°C and 42°C to study the effect of temperature. The effect of pH on production of LT was studied by using Mundell’s medium adjusted to pH 7.2, 7.8, 8.2, 8.6, 9.0 and 9.2. The effect of osmotic stress on LT production was determined by growing the bacteria in Mundells medium with different osmolarity (0.1, 0.2 and 0.3 M NaCl) and effect of nutrient limitation was determined by growing the bacteria in Mundells medium containing different concentrations of glucose (1, 2 and 2.5g/l).

The broth culture was incubated for 24 hours and centrifuged at 3000 g for 15 minutes. The supernatants were filtered through a 0.2 µm Millipore filter (Laxbro, Pune, India). The culture supernatants were used for estimation of LT using GM1 ELISA.

GM1-ELISA

GM1-ELISA to detect LT was performed essentially as previously described[6] with some modifications. ELISA microtiter wells were coated with GM1 (0.5 µg/ml in phosphate-buffered saline [PBS] at room temperature overnight), and the E. coli strains to be tested were grown in 100 µl of Luria Bertani broth (HiMedia Laboratories Pvt. Limited, Mumbai, INDIA) supplemented with lincomycin (45 µg/ml) and glucose (2.5 mg/ml) in GM1-coated microtiter wells at 37°C overnight. Released LT from the bacteria samples bound to the solid-phase GM1. This was followed by incubating the wells with rabbit anti-LT (Sigma-Aldrich, Missouri, USA) for 60 min at 37°C. Goat anti-rabbit immunoglobulin G alkaline phosphatase (Sigma-Aldrich) was added to the wells and incubated for 60 min at 37°C, and finally p-nitrophenyl phosphate (E. Merck, Mumbai, INDIA) was added as substrate. The reaction was stopped by adding 3N NaOH to the wells. The readings were taken at OD 405 using a spectrophotometer (Systronics, Naroda, INDIA).

In the light of the OD values obtained with 10 negative strains used in the screening GM1 ELISA, a net OD value 0.3 or greater was selected as cutoff for evidence of LT production.[7]

Statistical analysis

Kruskal-Wallis analysis of variants was used for comparison between the groups. A multiple comparison criterion test was used for pair wise comparisons.

Results

Of the four E. coli strains studied, the control strain of E. coli for LT production, MTCC 723 produced maximum amount of LT. All the four strains of E. coli produced maximum amount of LT (OD 3.285) when grown at 37°C followed by 40°C (OD 3.035) (Table 1). Least amount of LT was produced at 33°C. Growth of E. coli at 42°C suppressed LT production.

The pH of the growth medium had a significant effect on LT production by E. coli. All the four strains of E. coli produced least amount of LT when grown in medium having pH 7.2 (Fig. 1). In three strains of E. coli namely MTCC 723, S004 and S007, maximum amount of LT was produced when the bacteria were grown in medium with pH 8.6. In E. coli strain S001, the amount of LT produced was highest when grown in a medium of pH 8.2. The amount of LT produced by the four strains of E. coli when grown in medium of pH 9.0 was significantly less than (P<0.001) at pH 8.6.

In E. coli strains MTCC 723, S001 and S007 maximum amount of LT was produced in medium containing 0.2 M NaCl (Table 2). Growth of these strains in medium without NaCl and medium containing 0.1 and 0.3 M NaCl resulted in significantly less (P<0.01) amount of LT. E. coli strain S004 produced similar amounts of LT when grown in medium without NaCl and in medium containing 0.1 and 0.2 M NaCl. The amount of LT produced was least when bacteria were grown in a medium containing 0.3 M NaCl.

<table>
<thead>
<tr>
<th>E. coli strains</th>
<th>Temperature°C</th>
<th>OD405</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>33</td>
<td>37</td>
</tr>
<tr>
<td>MTCC 723</td>
<td>2.014 ± 0.125**</td>
<td>3.285 ± 0.108</td>
</tr>
<tr>
<td>S001</td>
<td>0.749 ± 0.096***</td>
<td>2.298 ± 0.105</td>
</tr>
<tr>
<td>S004</td>
<td>0.421 ± 0.051***</td>
<td>3.018 ± 0.123</td>
</tr>
<tr>
<td>S007</td>
<td>0.421 ± 0.051***</td>
<td>3.018 ± 0.123</td>
</tr>
</tbody>
</table>

*P < 0.05 **P < 0.01 ***P < 0.001
Maximum amount of LT (OD 3.150) was produced when the bacteria were grown in medium containing 2.5g/l of glucose (Table 3). Growth in medium containing less concentrations of glucose resulted in less LT production in all the four strains of *E. coli*. From the results, it is clear that optimum concentration of glucose for LT production is 2.5g/l of the medium.

**Discussion**

The present study was designed to investigate the effect of temperature, pH, osmotic pressure and nutrient limitation on production of LT by *E. coli*. All stress factors had a significant effect on the LT production by *E. coli*, though quantitative differences in the various strains were observed. Maximum amount of LT was produced at 37°C. Cells of all animals, plants and microorganisms studied so far display a transient synthesis of approximately two dozen proteins when exposed to temperatures above the optimum level for growth.[11] Higher concentration of these proteins is apparently needed for the cells to be able to grow at higher temperatures. It has been observed that, in *E. coli*, most of the virulence genes are optimally expressed at temperature slightly higher than the body temperature.[12] It has been shown that the optimum expression of virulence proteins in enteropathogenic *E. coli* (EPEC) correlated with natural body temperatures. EPEC proteins were detected at 33°C, but maximum levels were reached at 36°C. EPEC proteins were undetectable at 42°C.[13]

A previous study has shown that growth of organisms below 37°C results in decreased toxin production in *Bordetella pertussis*.[14] It has been postulated that growth temperature may act to affect a single gene or a group of genes.[14] These genes may in turn act directly or indirectly to control the expression of the ability of the organism to produce enterotoxin. The pH of the growth medium had a profound effect on the production of LT by *E. coli*. The results of the present study show that an alkaline pH 8.6 strongly favors LT production. ETEC, which produces LT, colonizes small intestine. Alkaline conditions of small intestine may favour LT production and contribute to pathogenesis of diarrhoea. The pH of the medium has been shown to have an effect on the protein secretion in EPEC.[15] While high levels of protein secretion were observed at pH between 6.35 and 7.6; secretion drastically reduced at pH 7.8 to 8.0.[15]

The medium osmolarity also had significant effect on LT production. We observed that *E. coli* grow within a narrow range of NaCl concentration of 0.1 to 0.3 M. This is in contrast to the findings of other workers who have shown the tolerance of *E. coli* to a wider range of NaCl.[16] *E. coli* cells shocked by addition of 0.2 M NaCl have been shown to resume growth at normal rates.[16] Higher concentrations of external osmolytes cause slow growth, implying that the mechanism of cellular accommodation to elevated osmotic strength is incomplete or it impairs the efficiency of metabolic processes.[17] In the present study, we used various concentrations of glucose to

### Table 2: Effect of osmotic stress on production of heat labile enterotoxin by *E. coli*

<table>
<thead>
<tr>
<th><em>E. coli</em> strains</th>
<th>Sodium chloride (M)</th>
<th>OD$_{405}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>MTCC 723</td>
<td>3.099 ± 0.062</td>
<td>1.709 ± 0.045***</td>
</tr>
<tr>
<td>S001</td>
<td>2.186 ± 0.139</td>
<td>2.579 ± 0.058*</td>
</tr>
<tr>
<td>S004</td>
<td>2.609 ± 0.145</td>
<td>2.736 ± 0.078</td>
</tr>
<tr>
<td>S007</td>
<td>1.634 ± 0.123</td>
<td>1.746 ± 0.083</td>
</tr>
</tbody>
</table>

*P <0.05, ***P <0.001

### Table 3: Effect of nutrient limitation on production of heat labile enterotoxin by *E. coli*

<table>
<thead>
<tr>
<th><em>E. coli</em> strains</th>
<th>Glucose (g/l)</th>
<th>OD$_{405}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>MTCC 723</td>
<td>3.150 ± 0.069</td>
<td>1.558 ± 0.314***</td>
</tr>
<tr>
<td>S001</td>
<td>2.241 ± 0.040</td>
<td>1.256 ± 0.271***</td>
</tr>
<tr>
<td>S004</td>
<td>2.664 ± 0.224</td>
<td>1.094 ± 0.078***</td>
</tr>
<tr>
<td>S007</td>
<td>1.642 ± 0.152</td>
<td>0.348 ± 0.086***</td>
</tr>
</tbody>
</table>

**P <0.01, ***P <0.001

Figure 1: Effect of pH on production of heat labile enterotoxin by *E. coli*

**Figure 1: Effect of pH on production of heat labile enterotoxin by *E. coli***
investigate the effect of nutrient limitation on the production of LT by *E. coli*. Maximum LT production was seen when the strains were grown in Mundell's casamino acids medium containing 0.25% glucose.

It has been suggested that environmental cues that signal the entry of microbe into the host tissues, may have a direct control on the expression of virulence factors by microorganisms.[12] Physicochemical properties such as temperature, osmolarity, pH, O₂, CO₂ or ions vary widely within different tissues of the host, the pathogen may use this as signal to detect anatomical differences in the human body.

The transition from ambient low temperature to body temperature has been correlated with dramatic changes in the expression of virulence determinants in several organisms. Transcription of virulence genes in several organisms like *Shigella* spp, *E. coli*, *Yersinia pestis*, *Pseudomonas* spp and *Bordetella* spp have been shown to be regulated by temperature.[18] The pathogenicity of many bacteria is said to be associated with the expression of specific genes.[19] Expression of such genes is highly regulated and responds to environmental stimuli, such as temperature, pH and nutrient availability.[19] Regulation of genes may permit the coordinated and timely expression of proteins required for growth and survival under changing environmental conditions.

The influence of growth temperature on the expression of virulence genes may prove to be a common characteristic of bacterial pathogens of mammals. The tight negative regulation of virulence genes and their subsequent expression in response to changes in environmental conditions are key elements in the pathogenic systems of many bacteria.[19] The observations made during the present study clearly show that environmental conditions profoundly affect the synthesis of heat labile enterotoxin by *E. coli*. This may have a direct effect on the pathogenesis of diarrhoea.

**References**


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