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Production of Enterotoxin by *Escherichia coli* at Four, Twenty-Two and Thirty-Seven Degrees Centigrade

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One hundred and seventy-seven *Escherichia coli* strains isolated from food, pigs and humans were tested for the production of heat-labile and heat-stable enterotoxin at 4, 22, and 37 °C. Heat-labile enterotoxin was detected in culture supernatants by an enzyme-linked immunosorbent assay, and heat-stable enterotoxin by the infant mouse bioassay. Thirty strains produced heat-labile enterotoxin, and twenty heat-stable enterotoxin. None of the strains isolated from food were enterotoxigenic. Fifty-seven per cent of the human and porcine strains producing heat-labile enterotoxin at 37 °C also produced the toxin at 4 °C. The fact that *Escherichia coli* enterotoxin may be present in food at consumption must be considered pathogenetically relevant.

Escherichia coli belongs to the normal intestinal flora, but may become enterotoxigenic through incorporation of a plasmid coding for enterotoxin production. *Escherichia coli* can produce two kinds of enterotoxin, either a heat-labile toxin or a low molecular heat-stable enterotoxin. Some strains produce both toxins simultaneously. These toxins are responsible for a cholera-like "rice-water" diarrhea, often called "tourist diarrhea" or "travellers diarrhea" (1).

Enterotoxigenic *Escherichia coli* has previously been regarded as a problem in food hygiene, and toxin-producing strains have been isolated from both food and water (2–6). Recently, another enterobacterial species, *Yersinia enterocolitica,* has been shown to produce heat-stable enterotoxin at 4, 22 and 37 °C (7, 8). This raises the question whether enterotoxigenic *Escherichia coli* may also cause diarrhea by ingestion of heat-labile or heat-stable enterotoxin produced in food and water at low temperatures.

Materials and Methods

Bacterial Strains. A total of 177 strains of *Escherichia coli* were examined for their ability to produce heat-labile and heat-stable enterotoxin at 4, 22, and 37 °C. The strains were divided into four groups according to origin.

One hundred and twenty strains were isolated from children with diarrhea in the Aker Hospital, Oslo, Norway. The strains underwent a maximum of three subcultures over a period of one week before cultivation for toxin production.

Twenty strains known to produce enterotoxins were kindly supplied by the Bureau of Epidemiology, Centers for Disease Control, Atlanta, USA. The strains had been isolated from patients with gastroenteritis then maintained on deep agar for one to two years.

Twenty strains of *Escherichia coli* were supplied by the Institute of Microbiology and Immunology, Veterinary College of Norway, Oslo, Norway. They were all collected from the jejunum of pigs which had died as a result of diarrhea. The strains were kept on deep agar for one to four months before toxin production.

Seventeen *Escherichia coli* strains isolated during routine food controls were provided by the Department of Food Hygiene, Veterinary College of Norway. The strains were isolated from bread, cakes, mussels, hamburgers, milk and water.

Growth Medium and Toxin Production. The strains were cultivated in 5 ml trypticase soy broth (Oxoid, London, England), the caps of the culture tubes being left partly open to allow aeration. The tubes were incubated in a roller drum (Bellco, Vineland, New Jersey, USA) at 60 rotations per minute in a walk-in incubator. Growth, measured as the number colony forming units (CFU) per ml on lactose agar plates, was

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recorded at various intervals for up to seven days. After incubation, the tubes were centrifuged for 30 min at 1000 *g* at 4 °C. The supernatants were then removed and passed through a membrane filter of pore size 0.45 µm to remove bacterial cells, then stored at −20 °C for one month before toxin detection. Enterotoxigenic supernatants did not loose their enterotoxin activity after storage at 4 °C for six months.

Detection of Enterotoxin. Heat-labile enterotoxin was assayed by a four-layer sandwich enzyme-linked immunosorbent assay (ELISA), employing burro anti-cholera enterotoxin as coating, rabbit anti-heat-labile enterotoxin in the third layer, and alkaline phosphatase labelled goat anti-rabbit IgG as conjugate (9). The colour intensity after enzyme reaction with the substrate, p-nitrophenylphosphate, was recorded photometrically at 410 nm. The rabbit ileal loop method (10) was used to demonstrate the enterotoxin activity biologically. Heat-stable enterotoxin was assayed using the infant mouse model (11). Supernatants were boiled for 5 min before injection directly into the stomach of three to four day old mice. The method of Kapperud (8) was used to distinguish positive from negative samples.

Polyacrylamide Gel Electrophoresis. Supernatants of three *Escherichia coli* strains of human and porcine origin cultivated at different temperatures were dialysed for five days at 4 °C with 0.1 mol/l phosphate buffered saline (PBS) of pH 7.2 containing 0.05 % (w/v) sodium azide. The supernatants were then concentrated 100-fold in an Amicon stirred cell system (Amicon Corporation, Lexington, Massachusetts, USA) with a PM-10 filter which allowed passage of all molecules less than 10,000 Dalton. The concentrate was dialyzed with 0.065 mol/l Tris-borate buffer of pH 9.0. Fifty µl of the concentrate containing enterotoxin was then placed on 7.5 % polyacrylamide gels (ratio of acrylamide to bis-acrylamid 32 : 0.8), 0.5 by 7 cm in size, in a model 155 electrophoresis apparatus (Bio-Rad Laboratories, Richmond, California, USA), with a 1 cm 4.5 % stacking gel.
Discontinuous electrophoresis was performed in a Tris-borate/Tris-sulphate buffer system of pH 9.0 (12). After stacking at 50 V for 30 min, the samples had entered the separating gel, and electrophoresis was performed at 2 mA/gel for 3 h at 10 °C.
Parallel gels were fixed in 12.5 % trichloroacetic acid and stained to detect protein by Coomassie Brilliant Blue R 250 (Bio-Rad Laboratories). Unfixed gels were cut into 2 mm slices for detection of heat-labile enterotoxin by ELISA. Each polyacrylamide gel slice was placed in an ELISA microtiter plate well containing 100 µl PBS with 0.05 % (w/v). Tween 20 (Technicon, Tarrytown, New York, USA). The ELISA plates were incubated overnight at room temperature in a humid atmosphere. The heat-labile enterotoxin was then dissolved in the PBS-Tween buffer and formed a bridge with specific antibodies in the coating layer. The gel slices were discarded and the wells washed thoroughly before performing the normal ELISA (9). The gels slices were emulsified in 1 ml PBS before bioassay.

Results

Enterotoxin Production

Table 1 shows that heat-labile and heat-stable enterotoxin was produced by 30 and 20 *Escherichia coli* strains respectively. Table 2 shows the ability of enterotoxigenic strains to produce enterotoxin at low temperatures. Of 30 strains producing heat-labile toxin at 37 °C, 17 also produced the toxin at 22 °C and 12 at 4 °C. Of 20 strains producing heat-stable toxin at 37 °C, eight also produced the toxin at 22 °C and three at 4 °C.

Table 1: Ability of *Escherichia coli* strains from various sources to produce enterotoxin at 37 °C.

Source strains	No. of strains	Heat-labile enterotoxin	Heat-stable enterotoxin
Humans (USA)	20	13	8
Children with diarrhea	120*	1	0
Pigs	20	16	12
Food	17	0	0
Total	177	30	20

* 17 strains were also tested at 4 and 22 °C.

All strains growing at 22 °C reached maximum growth after two days. By that time, both heat-labile and heat-stable enterotoxin production had reached maximum levels. Strains growing at 4 °C attained maximum levels of growth and heat-labile enterotoxin production after four days (Figure 1).

Table 2: Numbers of *Escherichia coli* strains producing heat-labile and heat-stable enterotoxins at 4, 22, and 37 °C.

Temperature	Heat-labile enterotoxin	Heat-stable enterotoxin
37 °C	30	20
22 °C	17	8
4 °C	12	3

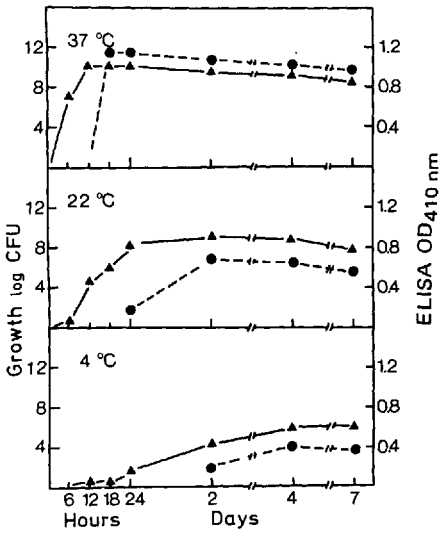

Figure 1: Growth (▲) and production (●) of heat-labile enterotoxin by *Escherichia coli* at 4, 22 and 37 °C. Heat-labile enterotoxin production was detected by ELISA.

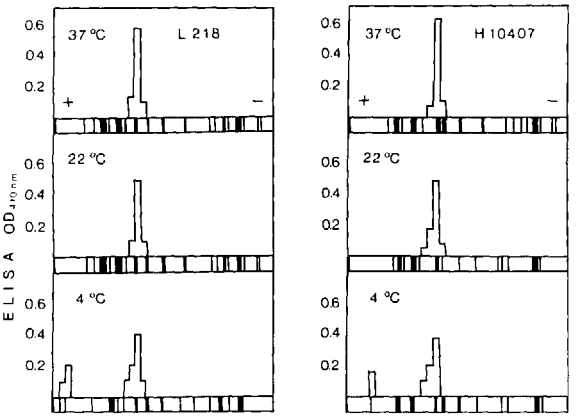

Figure 2: Electrophoresis band patterns of extracellular proteins produced by two enterotoxigenic *Escherichia coli* strains at 4, 22 and 37 °C. Gel slices were assayed by ELISA to detect heat-labile toxin. Strain H 10407 was of human origin, strain L 218 of porcine origin.

Strains which lost the ability to produce heat-labile and heat-stable toxin at lower temperatures were checked for loss of plasmid coding for enterotoxin production by recultivation of samples at 37 °C in fresh trypticase soy broth. None of these strains were found to have lost their enterotoxigenicity.

Identification of Heat-Labile Enterotoxin

Figure 2 shows that the electrophoretic mobility of the heat-labile enterotoxin bands were similar for selected strains at each incubation temperature. Only small differences were observed between porcine and human strains. Small additional bands in cultures grown at 4 °C showed a positive ELISA not attributable to complete heat-labile enterotoxin because enterotoxin activity had been absent in vivo.

Parallel to the ELISA method slices of the electrophoresis gels were also studied for their biological activity. Fluid secretion was elicited in rabbit ileal loops by gel slices corresponding to the major ELISA peaks, showing that these peaks represented active enterotoxin. Both the positive ELISA response and the ability to induce fluid secretion disappeared after the gel slices had been heated at 80 °C for 15 min. No liquid secretion was elicited by the minor peaks, however. These findings indicate that serological cross-reacting components were involved.

Discussion

Our findings show that *Escherichia coli* is capable of producing heat-labile and heat-stable enterotoxin at 4 and 22 °C, as well as at 37 °C. This parallels the patterns of enterotoxin production in *Yersinia enterocolitica* (7, 8) with one significant difference. In our study more *Escherichia coli* strains produced enterotoxin at 37 °C than at lower temperatures, whereas more *Yersinia enterocolitica* strains produce heat-stable enterotoxin at 22 °C than at either 4 or 37 °C. This difference between the enterotoxin production of *Escherichia coli* and *Yersinia enterocolitica* may be related to the different habitats and optimum growth temperature of the two species.

The properties of heat-labile enterotoxin from *Escherichia coli* appeared unrelated to the growth temperatures as shown by gel electrophoresis. It is important to note that only the major polyacrylamide gel bands containing ELISA positive material corresponded to complete heat-labile enterotoxin, irrespective of the growth temperature. Minor peaks showing ELISA positive material contained proteins which were antigenically related to heat-labile enterotoxin, but biologically inactive in the rabbit ileal loop system. It is therefore assumed that this fraction contains incomplete enterotoxin. Whether this moiety corresponds to the A or the B subunits of the complete enterotoxin, or to a combined but modified A + B fraction, remains to be determined.

Enterotoxin production was rare in isolates from Norwegian children with diarrhea. Only one of the 120 strains tested was positive for heat-labile enterotoxin, and none was positive for heat-stable enterotoxin. Enterotoxigenic *Escherichia coli* strains are not frequently isolated from humans in Norway in comparison to other European countries (13, 14).

Enterotoxin production was not observed in the 17 *Escherichia coli* strains isolated from food. The fact that enterotoxigenic strains have been isolated from food and water in other countries (2–6, 15, 16) indicates that toxin may be produced in food during storage. Benoki (17) reported in 1978 that *Escherichia coli* caused 3.8 % of all food poisoning in Japan. Our study indicates that enterotoxin can be found in contaminated food or water at temperatures as low as 4 °C. This is particularly important with respect to heat-stable enterotoxin which may remain biologically active even after bacterial inactivation.

Bacterial diarrhea is caused either by infection (e.g. salmonellosis, shigellosis, or invasive *Escherichia coli* enterocolitis), or by ingestion of preformed toxins, as is the case with enterotoxigenic *Staphylococcus aureus* (18) and *Bacillus cereus* (3). Bacteria such as *Vibrio cholerae*, enterotoxigenic *Clostridium perfringens* (19) and enterotoxigenic *Escherichia coli* (1) are pathogenic because they produce enterotoxin within the gastrointestinal tract. Enterotoxigenic *Escherichia coli* strains often adhere to intestinal epithelial cells by means of colonization pili (20, 21, 22). These pili, K88 or K99 (porcine strains) and CFA I or CFA II (human strains), play an important role in the pathogenicity of enterotoxigenic strains.

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