Heat stable enterotoxin produced by *Escherichia coli* in acute diarrhoea

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SUMMARY We screened 569 children with acute onset diarrhoea from five Italian towns for heat stable enterotoxin produced by *Escherichia coli*. We compared an enzyme linked immunoabsorbent assay (ELISA) with the standard suckling mouse assay for detection of the enterotoxin. A total of 31 (5.4%) children were found who had strains of *E coli* that produced heat stable enterotoxin: 26 strains were positive in both tests, four only in the suckling mouse assay, and one only in the ELISA. Compared with the suckling mouse assay the sensitivity of the ELISA was 87% and the specificity was 99.8%. The mean age of the children with *E coli* that produced heat stable enterotoxin was 22 months. Fifteen (48%) of the children had ingested potentially contaminated food and five (17%) had a previous contact with someone with diarrhoea. The clinical picture was milder than that described in developing countries. Faecal osmolality and the osmolal gap were consistent with a secretory diarrhoea in 12 out of 15 (80%) of these children. The mean duration of the diarrhoea was five days. Heat stable enterotoxin produced by *E coli* is a common cause of mild diarrhoeal illness in Italian children.

Diarrhoea induced by heat stable enterotoxin is the result of a multistep process involving (after small intestinal colonisation and release of heat stable enterotoxin¹) the binding of the toxin to receptors located on the brush border of enterocytes,² the activation of the guanylate cyclase-guanosine monophosphate (cGMP) system,³ and the resulting inhibition of sodium and chloride absorption along with the stimulation of anion and water secretion.⁴ Diarrhoea induced by heat stable enterotoxin is therefore of the secretory type, no signs of inflammation or colon involvement being usually present.⁵

Escherichia coli that produce heat stable enterotoxin are among the most common aetiological agents of diarrhoea in developing countries.⁶⁻¹⁰ Scanty information is available, however, about their role in industrialised countries. We have previously reported the isolation of *E coli* that produce heat stable enterotoxin in Naples, southern Italy, from less than 1% of children with acute gastroenteritis in two series of patients.^{11 12} A similar frequency has been reported in Brescia, northern Italy.¹³ Other investigators reported sporadic isolation from children with diarrhoea in

Finland,¹⁴ Canada,¹⁵ and the United States.¹⁶ No strains of *E coli* that produce heat stable enterotoxin have been found in Switzerland¹⁷ or Sweden.¹⁸

Thus in industrialised countries *E coli* that produce heat stable enterotoxin are regarded as putative agents of traveller's diarrhoea¹⁹ rather than as endemic intestinal pathogens. Accordingly, a search for these agents is not performed on a routine basis in patients with diarrhoea. The identification of heat stable enterotoxin produced by *E coli* is currently based on the suckling mouse assay,²⁰ which is reliable, does not require special facilities, is relatively inexpensive, but is cumbersome and time consuming. A possible alternative is the use of various DNA probes,²¹ which, however, require the use of radioisotopes and might give false positives.²² Thompson *et al* developed a competitive ELISA for heat stable enterotoxin which is rapid and does not require sophisticated equipment.²³ Until now this test has never been performed on a large number of patients.

As a part of a national investigation on the incidence, aetiology, diagnosis, and clinical features in childhood enteritis by the National Study Group on Acute Diarrhoea in Childhood (coordinated by

S Guandalini), we have screened 569 children with acute onset diarrhoea with the suckling mouse assay and the ELISA for heat stable enterotoxin produced by *E coli*. In the present report we focus on the epidemiological and clinical features of children with *E coli* that produce heat stable enterotoxin and the comparison between the suckling mouse assay and the ELISA for the diagnosis of diarrhoea related to heat stable enterotoxin.

Patients and methods

A total of 569 children (mean age 43 months, range: newborn to 15 years) from five towns in Italy (Milan, Mantua, Naples, Palermo, and Messina, table 1), who presented with diarrhoea of acute onset, were included in this study. Diarrhoea was defined as an unequivocal greater frequency or diminished consistency of stools, or both, in comparison with the previous usual pattern. Patients known to have received antibiotics were excluded. One hundred and seventy two children without gastrointestinal symptoms, and matched for age and season, were enrolled as controls. Clinical assessment was performed as previously described.¹²

The following information was collected from the parents by the physician in charge of each patient: (a) the presence of subjects with diarrhoea living in the same house in the two weeks before the onset of diarrhoea and (b) the ingestion of food by the patient known to be potentially contaminated and, specifically, uncooked mussels, eggs, and unwashed vegetables. All patients were drinking chlorinated tap water.

LABORATORY METHODS

Diarrhoea was considered in keeping with a secretory pattern when the osmolal gap [osmolality - 2 × (sodium + potassium concentration)] in the stool supernatant did not exceed 40 mmol/l. The isolation

and identification of enteropathogens (bacteria, viruses, and parasites) was performed as previously reported.²⁴ Five strains of *E coli* were pooled from each stool specimen of subjects as well as healthy controls. Strains to be tested for production of heat stable enterotoxin were stored in nutrient agar in the dark and sent to our laboratory in Naples. They were screened by the suckling mouse assay according to Giannella²⁰: strains were grown in casamino acid yeast extract in a shaker incubator, the cultures were centrifuged, and 0.1 ml was injected intragastrically in mice 2–4 days of age. After three hours the mice were killed by decapitation and the ratio from gut weight divided by body weight was determined. Values greater than 0.083 were considered positive; values less than 0.075 were considered negative; intermediate results were considered doubtful and repeated. *E coli* strain 214 Cl, which produces heat stable enterotoxin, and an *E coli* strain that does not served as controls.

The same supernatants were simultaneously screened for heat stable enterotoxin using an ELISA test as described by Thompson *et al.*²³ We used antiheat stable enterotoxin monoclonal antibodies prepared against pure heat stable enterotoxin from *E coli* strain 18 D.²⁵ The standard curve was obtained with heat stable enterotoxin purified by high performance liquid chromatography and quantitated by amino acid analysis. A total of 50 µl of heat stable enterotoxin-bovine serum albumin conjugate (1 µl/mg) in carbonate buffer, pH 9.6, were used to coat each well of a 96 well flat bottom microtitre plate (Limbro Eia Microtest, Flow Laboratories) as suggested.²³ After 18 hours of incubation at 4°C, the plates were washed and subsequently blocked with 1% bovine serum albumin in phosphate buffered saline for one hour at 37°C. An eight point standard curve of pure heat stable enterotoxin was added to each plate. Pure heat stable enterotoxin and samples were mixed with monoclonals (20 C1B8) in glass tubes on ice: each well received 50 µl of monoclonal antibody solution (final dilution 1:160 of 2 µg/ml stock solution) containing 5 µl of bacterial supernatant. After 18 hours of incubation at 4°C, the plates were washed and 50 µl of horseradish peroxidase labelled affinity purified goat antimouse IgG, at a final dilution of 1:250 in 0.1% bovine serum albumin in phosphate buffered saline, were added to each well for 15 minutes at room temperature. Plates were then rinsed and 100 µl of freshly diluted chromogenic substrate (2.2 azino-diethylbenzothiazoline sulphonic acid), with 0.03% hydrogen peroxide in 0.1M citrate buffer, pH 4, were added to each well. After an incubation of 20 minutes in the dark, plates were read by eye. Colour darker than the well containing 100 pg of

Table 1 Isolation rate of *E coli* that produce heat stable enterotoxin in five Italian towns

Town	No (%) children with <i>E coli</i> that produce heat stable enterotoxin/ No of children screened
Milan	19/201 (9.4)
Messina	1/14 (7.1)
Palermo	6/151 (3.9)
Naples	3/99 (3.0)
Mantua	2/104 (1.9)
Total	31/569 (5.4)
Controls	0/172

heat stable enterotoxin (corresponding to 20 ng of heat stable enterotoxin/ml of bacterial culture) were considered positive. Positive strains were tested twice in both the suckling mouse assay and the ELISA.

Both monoclonal antibodies and the heat stable enterotoxin were generous gifts of Dr R Giannella, Cincinnati, Ohio. Culture media were purchased from Difco and antimouse IgG from Kierkegard and Perry. All chemicals were of analytical reagent grade.

Statistical analysis was performed by χ^2 test and by the *t* test where appropriate.

Results

E coli strains were isolated from 541 of the 569 children with diarrhoea (95%) and from 150 of the 172 controls (87%). A total of 31 (5.4%) children, all belonging to the study group, had strains of *E coli* in their intestine that produced heat stable enterotoxin. These strains were isolated in each of the five towns in which the screening was performed (table 1).

The prevalence of strains of *E coli* that produce heat stable enterotoxin was 11% in children with infectious diarrhoea. They were the fourth most common infectious agent isolated from cases of intestinal infections after rotavirus (47%), campylobacter (17%), and salmonella (15%). In nine affected patients an associated pathogen was isolated: three had rotavirus, two salmonella, two campylobacter, one rotavirus and salmonella, and one rotavirus and campylobacter. A total of 26 strains of *E coli* were positive for heat stable enterotoxin in both ELISA and the suckling mouse assay, one only in the ELISA, four only in the suckling mouse assay (table 2). None of controls was positive for *E coli* that produce heat stable enterotoxin by either assay. Considering the suckling mouse assay as the standard test, specificity of the ELISA was 99.8% and sensitivity was 87%.

The seasonal pattern of *E coli* that produce heat

Figure Age distribution of children with *E coli* that produce heat stable enterotoxin (▨) and of all other patients (□). The prevalence of diarrhoea induced by heat stable enterotoxin ranged from 5.6% to 13.3% in first 3 years of life and decreased sharply thereafter.

stable enterotoxin was as follows: 13 (42%) cases in spring, five (16%) in summer, 12 (39%) in autumn, and one (3%) in winter. The age distribution of the affected children is shown in the figure: the mean age of affected children was 22 months (range 0–63 months) whereas the mean age of all the other children in the study group was 43 months (range 0–180 months) ($p < 0.05$). A possible interhuman spread of infection was recorded from six (20%) of the patients positive for *E coli* that produce heat stable enterotoxin and from 92 (17%) of all other children with diarrhoea ($p > 0.05$).

A total of 15 (48%) children with *E coli* that produce heat stable enterotoxin had previously ingested potentially contaminated food one week or less before the onset of symptoms compared with 118 (22%) of all other children enrolled ($p < 0.01$). Mean duration of diarrhoea was five days (median was 3.2); in 28 of the 31 children, however, symptomatology lasted less than seven days.

Two children were malnourished with weight below the 5th centile.

Clinical features of children with diarrhoea induced by heat stable enterotoxin produced by *E coli* where no other pathogens were found are listed in table 3 and compared with those of all other children belonging to the study group. The incidence of both dehydration and vomiting was significantly lower in children with *E coli* that produce heat stable enterotoxin than in all other patients. On the contrary, there were no differences in the presence of fever, abdominal pain, or associated respiratory symptoms.

In table 4 the stool characteristics of children

Table 2 Comparison of the ELISA and suckling mouse assay in detecting activity of heat stable enterotoxin

	ELISA positive	ELISA negative
Suckling mouse assay positive	26	4
Suckling mouse assay negative	1	660*

*Including 510 patients and 150 controls with *E coli* in the stools.

Table 3 Comparison of clinical features of children who were positive or negative for *E coli* that produce heat stable enterotoxin

	Positive No (%) (n=22)*	Negative No (%) (n=538)	p Value
Dehydration	4 (18)	306 (57)	<0.001
Fever (>38°C)	5 (23)	247 (46)	NS
Vomiting	3 (14)	253 (47)	<0.01
Abdominal pain	7 (32)	226 (42)	NS
'Cold' symptoms	6 (27)	199 (37)	NS

Nine children in whom *E coli* that produce heat stable enterotoxin were isolated together with other enteric pathogens were excluded.

Table 4 Comparison of stool characteristics of children who were positive or negative for *E coli* that produce heat stable enterotoxin

	Positive No (%) (n=22)*	Negative No (%) (n=538)	p Value
Watery	14 (64)	360 (67)	NS
Loose	8 (36)	178 (33)	NS
Gross blood	0 (0)	253 (47)	<0.001
Leucocytes	1 (5)	44 (13)	NS
Mucous	6 (27)	291 (54)	<0.05
Increased osmolal gap	12/15 (80)	Not performed	

*Nine children in whom *E coli* that produce heat stable enterotoxin were isolated together with other enteric pathogens were excluded.

positive for and negative for *E coli* that produce heat stable enterotoxin are reported: there were no remarkable differences in the two groups besides the higher incidence of gross blood in the stools of the children who were negative. Determination of sodium, potassium, and osmolality in faeces was performed in 15 subjects: a pattern consistent with secretory diarrhoea was detected in 12 children (80%).

All but five children received oral rehydration only: this consisted of glucose electrolyte (60 mmol sodium) solution. Only one child required intravenous correction of dehydration because of vomiting and severe acidosis.

Antibiotics were given to four children. All patients did well, however, and rapidly regained weight after recovery from the diarrhoea.

In three children the diarrhoea became chronic (>14 days) and the presence of *E coli* that produce heat stable enterotoxin was persistent in the stools, but it ceased spontaneously.

Discussion

Our data suggest that heat stable enterotoxins

produced by *E coli* are a common cause of diarrhoea in paediatric patients in Italy, and that the relative frequency is presently increasing in comparison with the previously available information.¹¹⁻¹³ The decreasing incidence reported in the total number of cases caused by other better known pathogens (probably because of better prevention) is possibly responsible, at least in part, for the observed relative increase in the incidence of diarrhoea caused by heat stable enterotoxin in our country.

Strains of *E coli* that produce heat stable enterotoxin were isolated from all five towns in which the investigation was carried out; this suggests that it is endemic in Italy. No outbreaks occurred during the study period. The history of previous ingestion of potentially contaminated food in about 50% of the affected children suggests that food is likely to be the predominant source of infection. This assumption is supported by previous reports of the role of contaminated food in the endemic and the epidemic spread of enteritis caused by heat stable enterotoxin produced by *E coli*.²⁶⁻²⁸

Transmission through contacts who had diarrhoea is thought to be rare, as a large inoculum is apparently necessary to induce diarrhoea.²⁹ We have no clear evidence of such a transmission in our series as we did not attempt to isolate *E coli* that produce heat stable enterotoxin from potential contacts. The record of contacts with subjects who had diarrhoea, in any event, in affected patients was similar to that of all children enrolled in the study group.

Spring and autumn peaks in detection were different from that usually reported in developing countries, where the prevalence of isolation is found in the warm and humid season.²⁷

The mean age of affected children was younger than that of the study group. The increased susceptibility of young infants to diarrhoea induced by heat stable enterotoxin has been previously reported in developing countries.^{6,30} The higher sensitivity of young children to the enterotoxin is supported by recent laboratory investigations. Cohen *et al* have in fact shown in an animal model that the number of receptors for heat stable enterotoxin is increased in young subjects.³¹ One of the authors (AG) has reported that in children heat stable enterotoxin produced by *E coli* induces an increase in guanylate cyclase activity, whose magnitude is maximal in the newborn and then decreases with an age related pattern.³² Therefore the increased susceptibility and severity observed seems to be related to the state of intestinal development.

The overall clinical picture of diarrhoea induced by heat stable enterotoxin was that of a mild to moderate diarrhoea, far less severe than that

reported in developing countries. Furthermore, the incidence of dehydration was significantly lower in patients with heat stable enterotoxin produced by *E coli* than in patients with other diagnosis. This was somehow surprising in view of the present understanding of the pathophysiology of diarrhoea mediated by heat stable enterotoxin as a diarrhoea of the secretory type.³³ It should be noted, however, that all patients, with one exception, did well with oral rehydration only. The effectiveness of oral solution in preventing dehydration was due, at least in part, to its prompt administration soon after the onset of diarrhoea. In this respect it is of interest to note that values of faecal sodium and potassium and osmolality were consistent with a secretory diarrhoea in about 80% of our patients.

Mean duration of symptomatology in our study group was not different from that reported in developing countries,^{6,34} despite the different incidence of malnutrition in the two population samples. Three children had a protracted diarrhoea, which in one case lasted longer than one month. This unusual presentation of diarrhoea induced by heat stable enterotoxin suggests that the enterotoxin should be considered in the differential diagnosis of chronic diarrhoea.

The reported mortality rate associated with diarrhoea caused by heat stable enterotoxin is variable in developing countries.³⁵ All of our patients did well thus suggesting that diarrhoea induced by the enterotoxin: (a) does not itself have a high virulence, (b) can be effectively counteracted by prompt rehydration, and (c) is probably less severe in well nourished children.

The ELISA test used was rapid, easy to perform, reliable, and did not require sophisticated equipment. Four strains of *E coli* that produce heat stable enterotoxin were not detected by the ELISA whereas one was positive in the ELISA only. We have characterised an enterotoxin produced by *Klebsiella pneumoniae*, which behaves like the heat stable enterotoxin produced by *E coli*, but is not detected by the ELISA (manuscript in preparation). It is therefore possible that some differences in the primary sequence of the family of heat stable enterotoxins could affect their detection by monoclonal antibodies raised against pure heat stable enterotoxin from *E coli*. We do not have an explanation for the strain that was positive exclusively in the ELISA.

Even though the ELISA test did not detect about 10% of the heat stable enterotoxins detected by the suckling mouse assay, we believe that this is a good alternative to the latter and to the DNA probe, particularly when large numbers of samples are to

be screened or if animals or radioisotopes are not available.

Conclusion

Heat stable enterotoxin produced by *E coli* is a common cause of acute watery diarrhoea in Italy. Contaminated food is likely to be the main source of infection. Younger infants are more susceptible to diarrhoea caused by heat stable enterotoxin produced by *E coli*. The symptomatology is usually milder in Italy than that described in developing countries. The symptomatology is milder than that associated with other aetiologies. Heat stable enterotoxin can be responsible for chronic diarrhoea. The ELISA test with monoclonal antibodies is a convenient and reliable tool for diagnosis.

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