Comparison of rapid methods for detection of heat-labile (LT) and heat-stable (ST) enterotoxin in *Escherichia coli*

ÉVA CZIRÓK, G. SEMJÉN*, H. STEINRÜCK†, MARIA HERPAY, HEDDA MILCH, ILDIKO NYOMÁRKAY‡, ZSOFIA STVERTECZKY§ and AGNES SZENESS||

National Institute of Hygiene, "Bela Johan", Gyáli u 2–4, H-1966 Budapest, *University of Veterinary Science, POB H-1400 Budapest, †Institut für Infektionskrankheiten i, Kindesalter Berlin-Buch, O-1115 Berlin-Buch, Germany, ‡Public Health Station H-1138 Budapest, §Pest County Public Health Station, H-1032 Budapest and ||Győr-Ménfőcsanak Sopron County Public Health Station, H-9024 Győr, Hungary

Summary. Oxoid VET-RPLA, ST-EIA and Pharmacia Phadebact ETEC-LT enterotoxin tests were compared to find a simple but reliable method for detecting enterotoxigenic *Escherichia coli* (ETEC) in Hungary. In the Oxoid tests, all six reference LT- or ST-producing strains, except one ST-producer, gave positive results. Of 11 reference porcine enterotoxigenic strains, all four LT-producers gave positive reactions for LT but three of 10 ST-producers gave negative reactions for ST. Thirteen of 50 strains from culture collections of H. Steinrück (Germany) were LT⁺ and nine of 33 were ST⁺. When 31 isolates were tested simultaneously with the Oxoid and the Pharmacia LT tests, 12 strains were LT⁺ by the Oxoid LT test but by the Phadebact LT test only seven of these strains were LT⁺ and, of the remainder, three gave uncertain results and two gave negative results. Of 69 porcine strains, seven were LT⁺ and three ST⁺. Of 901 human strains isolated in Hungary, 10 were LT⁺ and one of 24 tested was ST⁺. In two cases, ETEC strains were isolated from contacts of travellers returning from Mongolia and Bangladesh. Results of comparative studies with reference strains corresponded well to those of the classical toxin detection tests. The Oxoid test was rapid, sensitive, specific and easy to perform and is recommended for use in screening ETEC isolates.

Introduction

Escherichia coli is a common commensal organism in the gut, where most strains are non-pathogenic. However, some can produce diarrhoea by a number of distinct mechanisms. Based on virulence properties, interactions with the intestinal mucosa, clinical symptoms and O:H serotypes, diarrhoeagenic *E. coli* strains can be divided into four categories: enterotoxigenic (ETEC), entero-invasive (EIEC), enteropathogenic (EPEC) and enterohaemorrhagic (EHEC).¹

ETEC are an important cause of diarrhoeal disease in man and animals.^{2–4} In man, ETEC are encountered in tropical areas as endemic pathogens and they are also the agents most frequently responsible for travellers' diarrhoea. Among newborn calves and piglets, scouring elicited by ETEC is also common and causes serious economic losses.

ETEC strains can produce two types of enterotoxins³—LT, a heat-labile enterotoxin⁵ and ST, a heat-stable enterotoxin.^{6,7} Two antigenically different forms of LT—LTI and LTII—are known.⁸ There

are also two types of ST—STa is active in a suckling mouse model,⁹ whereas STb causes fluid accumulation in the ligated intestinal loop of pigs but is inactive in the suckling mouse test.¹⁰ There are two forms of STa—STa1 and STa2.¹¹

Several methods have been developed for the detection of enterotoxin: the rabbit ligated-intestinal loop model;^{12,13} the rabbit skin test;¹⁴ the Y-1 adrenal cell assay;¹⁵ and the enzyme-linked immunosorbent assay (ELISA).¹⁶ However, none of these proved to be suitable for screening for enterotoxigenic *E. coli* strains in routine bacteriological laboratories to determine their incidence.

Recently,^{17,18} some new tests (Oxoid toxin detection kits, Pharmacia Phadebact ETEC-LT test) have become available and offer the possibility of rapid, simple, detection of enterotoxigenic *E. coli*. The Oxoid tests are suitable for the detection of LTI- as well as STa1- and STa2-producing *E. coli*.¹¹

Our aim was to compare the accuracy, sensitivity and specificity of these new tests, and to choose the simplest and most reliable one for screening for enterotoxin producing *E. coli* strains and determining their incidence in Hungary.

Materials and methods

Bacterial strains

A total of 1042 strains was studied. Porcine strains (69) were isolated at a farm where postweaning diarrhoea occurred frequently. The human strains (873) originated from diarrhoeal cases and were isolated during 1981–1983 and in 1990; another 28 strains were isolated by Milch and Gyenes¹⁹ in 1972. In addition, 11 reference strains and strains from the culture collections of H. Steinrück (50 strains) and G. Semjén (11 strains) were used for comparative studies. The latter were tested by Y-1 cell culture, the ligated loop test or the suckling mouse model. All 1042 strains were examined for LT and 80 for ST production by Oxoid tests. Comparative studies of 31 strains were made with the Pharmacia LT and Oxoid LT tests.

Enterotoxin assays

For detection of LT production, the Oxoid toxin detection kit (VET-RPLA; based on reversed passive latex agglutination) and the Phadebact ETEC-LT test (based on coagglutination) (Pharmacia Fine Chemicals, Uppsala, Sweden) were used according to the instructions of the manufacturers. STa was detected by the Oxoid ST-EIA test based on a competitive enzyme immunoassay. Briefly, for the Oxoid LT test, *E. coli* isolates were inoculated into Mundell's medium—Casein Peptone (Oxoid L42) yeast extract, salt solution (ferric chloride, manganese chloride, magnesium sulphate)—and incubated at 37°C for 18 h. Polymyxin B was added to give a concentration of 10 000 units/ml. After further incubation at 37°C for 4 h the culture was centrifuged at 900 *g* for 20 min and the supernate was used as the test sample. For ST detection, strains were inoculated into Casein-Yeast (CA-YE) broth, incubated at 37°C for 18 h and the cultures were centrifuged at 900 *g* for 30 min. The supernate was used as the test sample. For the

Phadebact LT test, strains were grown on blood agar and a loopful was suspended in 100 µl of "extraction solution" for the test sample.

Cell culture assay

The cytotoxic activity of strains on Vero cells was studied according to the method previously described.²⁰

Results

All the reference LT- and ST-producing strains, except strain P307 which failed to produce ST, were found by the Oxoid tests to produce the expected toxin (table I). Verotoxin-producing *E. coli* and *Shigella dysenteriae* serovar 1 strains were found to be enterotoxin-negative. *E. coli* strains 3 and 4 of serotype O26:H11, reported to be enterotoxigenic,²⁷ were found by us to produce verotoxin and they produced neither LT nor ST by the Oxoid tests.

When 11 (Semjén's) porcine enterotoxigenic strains were tested, all four LT-producers gave positive reactions in the Oxoid tests for LT (table II). Of the 10 ST-producing strains (strains whose soft agar culture supernates after heating at 100°C for 30 min caused fluid accumulation in the ligated gut of 5-week-old pigs), seven gave positive results and three gave negative results in the Oxoid ST test. In both tests, no reaction was elicited by the non-enterotoxigenic (P70) strain.

To compare the Oxoid LT (VET-RPLA) and the Pharmacia LT (Phadebact ETEC-LT) tests, 31 isolates were tested simultaneously (table III). By the Oxoid test 12 strains were found to be LT-positive, whereas with the Phadebact test only seven of these strains gave positive results, three gave uncertain results and two were negative. In addition, two Oxoid test-negative strains gave uncertain reactions by the Pharmacia test.

Table I. The results obtained with reference strains with Oxoid toxin detection kits

Strain designation	Reference	Serogroup or serotype of strain	Type of toxin produced	Reaction in the tests	
				LT (titre)	ST (OD)
PB176	21	<i>E. coli</i> O6:H16	LT, ST	+(1:32)	+(0.200)
P307	23, 24	O8:K87, 88	LT, ST*	NT	-(1.083)
P16	22	O9:K?	ST	NT	+(0.188)
H30	20	O26:H11	VT	-($<1:2$)	-(1.010)
H10407	25	O78:H11	LT, ST	+(1:64)	+(0.140)
E57	26	O138:K81	ST	NT	+(0.163)
P155	24	O149:K91, 88ac	LT, ST	+(1:32)	+(0.159)
337-85	W	O157:H7	VT	-($<1:2$)	-(1.108)
904	S	<i>S. dysenteriae</i> 1	VT	-($<1:2$)	-(1.084)
3†	27	<i>E. coli</i> O26:H11	LT	-($<1:2$)	-(1.115)
4†	27	O26:H11	LT	-($<1:2$)	-(0.809)

*STb producer.

†VT⁺ by our examinations.

OD, optical density; NT, not tested; W, Wachsmuth I K, haemorrhagic colitis, sporadic case, California, CDC Atlanta (personal communication); S, Solodovnikov Yu P, Enteritis, Nicaragua (personal communication).

Table II. Enterotoxin detection in porcine strains*

Strain designation	Serogroup or serotype of strain	Ligated loop test		Oxoid test	
		LT	ST	LT	ST
SWP99†	O141:K85ab, 88ab	+	+	+	+
P49†	O138:K81, 88ac	+	+	+	—
P9	O149:K91, 88ac	+	+	+	+
P30	O141:K88ac	+	+	+	—
P58	O115:K.	—	+	—	+
P783	O149:K91	—	+	—	+
P862	O138:K81	—	+	—	+
SWP105	O141:K85	—	+	—	+
SWP3	O139:K82	—	+	—	—
P70	O141:K85ab	—	—	—	—
P54	O138:K81	—	+	—	+

+, toxin formed; —, toxin not formed.

* From culture collection of G. Semjén.

† See ref. 28 for P, and 29, 30 for SWP strains.

Thirteen out of 50 German strains (Steinrück collection) were LT⁺ and nine out of 33 were ST⁺ by the Oxoid test (table IV). Eight of these also produced LT and one produced ST only. All but one of the strains found to be LT⁺ when tested in Germany were also positive by the Oxoid test, but seven strains found

Table III. Enterotoxin production by 31 isolates of *E. coli* determined by Oxoid VET-RPLA and Phadebact ETEC-LT tests

Results	Number of strains
Both tests negative	17
Both tests positive	7
Oxoid positive, Phadebact dubious	3
Oxoid positive, Phadebact negative	2
Oxoid negative, Phadebact dubious	2

Table IV. Comparative studies for enterotoxin detection in *E. coli* strains originating from Germany*

Oxoid toxin test result	Number of strains that were			
	LT† (50)		ST† (33)	
	+(14)	—(36)	+(15)	—(18)
VET-RPLA (LT) titre				
0	1‡	36	...	...
4	4	0	...	...
8	4	0	...	...
16	4	0	...	...
64	1	0	...	...
4-64 (+)	13	0	...	...
ST-EIA (ST)				
+	...	...	8	1§
—	...	...	7§	17

* From culture collections of H. Steinrück.

† Tests done in Germany (Y1 and CHO culture or suckling mouse test).

‡ Repeated examination by VET-RPLA gave a negative result (strain probably lost its original toxicity).

§ Repeat tests gave negative results in suckling mouse test (strains probably lost their original toxicity).

two years earlier in Germany to be ST⁺ were negative by the Oxoid test. However, it could not be excluded that these strains had lost their original toxicity.

Of the 901 human strains isolated in Hungary from faecal samples or jejunal juice of patients with gastroenteritis, 10 strains, one a strain of *Citrobacter freundii*, were found to produce LT and none of these produced ST. Six of these were amongst recent (1990) isolates from 300 patients. Of 24 strains tested for ST, one was ST⁺, but LT[—] (table V). In two cases, ETEC were isolated from contacts of travellers who had been visiting Bangladesh and Mongolia.

Of the 69 pig strains, seven produced LT only (these were of serogroup O8, O141 and O149) and three produced ST only (these were serogroup O147 and O157).

Discussion

Infantile diarrhoea through enterotoxigenic *E. coli* strains is rare in developed countries, but occasional outbreaks have been reported.³¹ Although ETEC strains from widely separated geographic areas belong to a comparatively limited number of serogroups,³² the serogroups commonly associated with ETEC (e.g., O6, O8, O20, O25, O78, etc) are also the ones occurring most frequently in the faeces of healthy individuals.³³ Therefore, serotyping isolates is an unsatisfactory method of detecting ETEC. The only way to detect ETEC is to show LT and/or ST production. Because LT is structurally, antigenically and functionally related to cholera toxin,³⁴ detection is based on cholera toxin detection methods. However, these are too complicated and time-consuming for regular use in non-specialist laboratories.

Now rapid and simple tests for enterotoxins based on agglutination are available commercially.^{35,36} Through their use it was shown that ETEC was more commonly associated with diarrhoeal disease in Australia than had been anticipated.³⁷ On the other

Table V. Enterotoxigenic isolates from human cases of enteritis in Hungary

Strain designation	Year of isolation	Organism, serotype	Patient age, source	Oxid toxin detection tests	
				LT (titre)	ST
31/5	1979	<i>C. freundii</i>	4m, Faeces	1:8	—
104918	1981	<i>E. coli</i> O15:H25	3y, Faeces	1:32	—
734	1983	O20:H—	7y, jejunal juice	1:64	—
100007	1982	O20:H—	5y, Faeces	—	+
95366	1982	O159:H4	2y, Faeces	1:32	—
511	1990	O7:HNT	60y, Faeces	1:8	—
593	1990	O6:H—	3y, Faeces	1:32	—
635	1990	O7:HNT	13y, Faeces*	1:16	—
636	1990	O6:H16	5y, Faeces	1:8	—
637	1990	O6:H16	4y, Faeces	1:8	—
668	1990	O6:H16	27y, Faeces†	1:4	—

+, Toxin detected; —, no toxin detected; NT, not typable.

* Patient's father recently returned from Bangladesh.

† Patient's husband returned from Mongolia where he had enteritis.

hand, Scotland *et al.*³⁸ recommended the Oxoid LT test for detecting the presence of LT⁺ strains because of its high specificity, ease of performance and speed. Almeida *et al.*¹⁸ examined *V. cholerae* enterotoxigenic strains by this test and supported this view.

When the Oxoid LT test and the Phadebact LT test were compared, the former proved to be more sensitive. In some cases, in addition to falsely negative results, the Phadebact test gave unclear results. However, the results of the Oxoid test on the reference enterotoxigenic strains agreed with those determined by other assays. On the basis of our findings, we recommend use of Oxoid tests, because they are rapid, sensitive, specific and easy to perform.

The Oxoid test was less useful in detecting ST-producing *E. coli* among porcine isolates.³⁹ Moreover, as this test uses plates with precoated wells, it is not an efficient method for testing small numbers of isolates from sporadic cases.

Our results also show that the Oxoid LT and ST tests can differentiate LT and ST production from production of other cytotoxins. As expected, strain

H30 of serotype O26:H11 and a VT-producer²⁰ gave negative results in tests for ST and LT. Previous tests,^{13,27,40} in which VT-producers were tested in animal models, gave inconsistent results although it has been shown that purified VT is active in the rabbit ileal loop model. In this study we found that strains 3 and 4, also of serotype O26:H11 and previously considered to produce an altered LT^{27,41} gave negative results for LT and ST by the Oxoid tests but positive results in a test for VT. Thus, the specificity of Oxoid LT and ST tests can preclude such confusions in classification of ST, LT or VT.

This work has revealed that LT-producing strains are also involved in human diarrhoeal disease in Hungary. The detection of an LT-producing *C. freundii* strain is interesting, particularly because our recent examinations of an outbreak of diarrhoea showed ST⁺ strains of *C. freundii* to be the aetiological agent. Our public health laboratories have started to search for toxigenic isolates in diarrhoeal epidemics of unknown origin by the rapid, simple Oxoid tests.

References

- Levine MM. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroadherent. *J Infect Dis* 1987; **155**: 377–389.
- Gorbach SL, Banwell JG, Chatterjee BD, Jacobs B, Sack RB. Acute undifferentiated human diarrhea in the tropics. *J Clin Invest* 1971; **50**: 881–889.
- Smith HW, Gyles CL. The relationship between two apparently different enterotoxins produced by enteropathogenic strains of *Escherichia coli* of porcine origin. *J Med Microbiol* 1970; **3**: 387–401.
- Sack DA, Kaminsky DC, Sack RB *et al.* Prophylactic doxycycline for travellers' diarrhea. Results of a prospective double-blind study of peace corps volunteers in Kenya. *N Engl J Med* 1978; **298**: 758–763.
- Evans DJ, Chen LC, Curlin GT, Evans DG. Stimulation of adenyl cyclase by *Escherichia coli* enterotoxin. *Nature New Biol* 1972; **236**: 137–138.
- Aldrete JF, Robertson DC. Purification and chemical characterization of *Escherichia coli* heat-stable enterotoxin. Annual Meeting of the American Society for Microbiology. 1977; 33, Abstract no. B105.
- Pesti L, Semjén G. *Escherichia coli* vaccine for prevention of neonatal enteric colibacillosis in pigs. *Acta Vet Acad Sci Hung* 1980; **28**: 1–11.
- Holmes RK, Twiddy EM, Pickett CL. Purification and characterization of type II heat-labile enterotoxin of *Escherichia coli*. *Infect Immun* 1986; **53**: 464–473.
- Burgess MN, Bywater RJ, Cowley CM, Mullan NA, Newsome PM. Biological evaluation of a methanol-soluble, heat-stable *Escherichia coli* enterotoxin in infant mice, pigs, rabbits and calves. *Infect Immun* 1978; **21**: 526–531.
- Greenberg RN, Guerrant RL. *Escherichia coli* heat-stable enterotoxin. *Pharmacol Ther* 1981; **13**: 507–531.
- Scotland SM, Willshaw GA, Said B, Smith HR, Rowe B. Identification of *Escherichia coli* that produces heat-stable enterotoxin ST_a by a commercially available enzyme-linked immunoassay and comparison of the assay with infant mouse and DNA probe tests. *J Clin Microbiol* 1989; **27**: 1697–1699.

12. De SN, Bhattacharya K, Sarkar JK. A study of the pathogenicity of strains of *Bacterium coli* from acute and chronic enteritis. *J Pathol Bacteriol* 1956; **71**: 201–209.
13. Taylor J, Bettelheim KA. The action of chloroform-killed suspensions of enteropathogenic *Escherichia coli* on ligated rabbit-gut segments. *J Gen Microbiol* 1966; **42**: 309–313.
14. Craig JP. A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. *Nature* 1965; **207**: 614–616.
15. Sack DA, Sack RB. Test for enterotoxigenic *Escherichia coli* using Y1 adrenal cells in miniculture. *Infect Immun* 1975; **11**: 334–336.
16. Yolken RH, Greenberg HB, Merson MH, Sack RB, Kapikian AZ. Enzyme-linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. *J Clin Microbiol* 1977; **6**: 439–444.
17. Bettelheim KA, Gracey M, Wadström T. The use of coagglutination test to determine whether Australian and New Zealand isolates of *Escherichia coli* produce the heat-labile enterotoxin. *Zentralbl Bakteriell Hyg A* 1985; **260**: 293–299.
18. Almeida RJ, Hickman-Brenner FW, Sowers EG, Puhf ND, Farmer JJ, Wachsmuth IK. Comparison of a latex agglutination assay and an enzyme-linked immunosorbent assay for detecting cholera toxin. *J Clin Microbiol* 1990; **28**: 128–130.
19. Milch H, Gyenes M. Subdivision and correlation studies of serologically grouped *Escherichia coli* strains by phage typing, colicinogeny, lysogeny and biochemical test. *Acta Microbiol Acad Sci Hung* 1972; **19**: 213–244.
20. Konowalchuk J, Speirs JJ, Stavric S. Vero response to a cytotoxin of *Escherichia coli*. *Infect Immun* 1977; **18**: 775–779.
21. Evans DG, Evans DJ. New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups O6 and O8. *Infect Immun* 1978; **21**: 638–647.
22. Gyles CL. Heat-labile and heat-stable forms of the enterotoxin from *E. coli* strains enteropathogenic for pigs. *Ann N Y Acad Sci* 1971; **176**: 314–322.
23. Lee CH, Moseley SL, Moon HW, Whipps SC, Gyles CL, So M. Characterization of the gene encoding heat-stable toxin II and preliminary molecular epidemiological studies of enterotoxigenic *Escherichia coli* heat-stable toxin II producers. *Infect Immun* 1983; **42**: 264–268.
24. Gyles CL. Immunological study of heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae*. *Infect Immun* 1974; **9**: 564–570.
25. Evans DJ, Evans DG. Three characteristics associated with enterotoxigenic *Escherichia coli* isolated from man. *Infect Immun* 1973; **8**: 322–328.
26. Sojka WJ. Enteropathogenic *Escherichia coli* in man and farm animals. Symposium on microbial food-borne infections and intoxications, Ottawa. 1972: 5–17.
27. Kétyi I, Czirók É, Vertényi A, Málovics I, Pácsa S. Comparison of *Escherichia coli* enterotoxin tests. *Acta Microbiol Acad Sci Hung* 1978; **25**: 23–36.
28. Pesti L, Semjén G. Studies on enteropathogenicity, loop dilating effect and enterotoxin producing capacity of *Escherichia coli* strains isolated from enteric diseases of swine. *Acta Vet Acad Sci Hung* 1973; **23**: 227–236.
29. Smith HW, Halls S. Observations by the ligated intestinal segment and oral inoculation methods on *Escherichia coli* infections in pigs, calves, lambs and rabbits. *J Pathol Bacteriol* 1967; **93**: 499–529.
30. Smith HW, Gyles CL. The effect of cell-free fluids prepared from cultures of human and animal enteropathogenic strains of *Escherichia coli* on ligated intestinal segments of rabbits and pigs. *J Med Microbiol* 1970; **3**: 403–409.
31. Ryder RW, Wachsmuth IK, Buxton AE et al. Infantile diarrhea produced by heat-stable enterotoxigenic *Escherichia coli*. *N Engl J Med* 1976; **295**: 849–853.
32. Ørskov F, Ørskov I, Evans DJ, Sack RB, Sack DA, Wadström T. Special *Escherichia coli* serotypes among enterotoxigenic strains from diarrhoea in adults and children. *Med Microbiol Immunol (Berlin)* 1976; **162**: 73–80.
33. Czirók É, Martön A, Csik M, Solt M. Virulence factors of *Escherichia coli* I. Mannose resistant haemagglutinating capacity is associated with serogroup but not with site of infection. *Acta Microbiol Hung* 1984; **31**: 187–195.
34. Field M. Modes of action of enterotoxins from *Vibrio cholerae* and *Escherichia coli*. *Rev Infect Dis* 1979; **1**: 918–925.
35. Rönnberg B, Wadström T. Rapid detection by a coagglutination test of heat-labile enterotoxin in cell lysates from blood agar-grown *Escherichia coli*. *J Clin Microbiol* 1983; **17**: 1021–1025.
36. Finkelstein RA, Yang Z. Rapid test for identification of heat-labile enterotoxin-producing *Escherichia coli* colonies. *J Clin Microbiol* 1983; **18**: 23–28.
37. Bettelheim KA, Hanna N, Smith DL, Dwyer BW. Evaluation of the Phadebact ETEC-LT test for heat-labile enterotoxin of *Escherichia coli*. *Zentral Bakteriell* 1989; **271**: 70–76.
38. Scotland SM, Flomen RH, Rowe B. Evaluation of a reversed passive latex agglutination test for the detection of *Escherichia coli* heat-labile toxin in culture supernatants. *J Clin Microbiol* 1989; **27**: 339–340.
39. Whipp SC. Protease degradation of *Escherichia coli* heat-stable, mouse-negative, pig-positive enterotoxin. *Infect Immun* 1987; **55**: 2057–2060.
40. Evans DG, Evans DJ, Gorbach SL. Identification of enterotoxigenic *Escherichia coli* and serum antitoxin activity by the vascular permeability factor assay. *Infect Immun* 1973; **8**: 731–735.
41. Kétyi I, Emödy L, Pácsa S, Vertényi A, Kontrohr T. An altered heat-labile enterotoxin (LT') produced by *Escherichia coli* serogroup O55 strain. *Acta Microbiol Acad Sci Hung* 1979; **26**: 255–262.