

**Toxin production and haemagglutination in strains of
Escherichia coli from diarrhoea in Brescia, Italy**

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SUMMARY

Two hundred and ninety-nine different strains of *Escherichia coli*, isolated from 172 patients with diarrhoea and 113 healthy subjects, were examined for enterotoxin, cytotoxin and haemolysin (Hly) production and for mannose-resistant haemagglutination (MRHA) and invasive properties. Three strains proved enterotoxigenic, none enteroinvasive; cytotoxin and Hly production was shown in 25 strains from patients and in 3 from controls. Ten strains produced the cytotoxic necrotizing factor (CNF), 6 released other factors which kill cell cultures. Hly production was shown in 21 strains, 9 of which were also positive for CNF. MRHA was detected in 26% of strains from diarrhoea compared with 14% of strains from healthy people. A strong association between toxin production and MRHA was demonstrated. Serotyping results showed that the strains exhibiting virulence traits mostly belonged to serogroups commonly involved in extra-intestinal infections. The possible role of strains of *E. coli* showing one or more virulence factors as opportunistic pathogens in diarrhoeal diseases is discussed.

INTRODUCTION

Rowe (1970) has reviewed the evidence that *Escherichia coli* can cause diarrhoea by the production of enterotoxins. A possible involvement in the pathogenesis of diarrhoea has also been suggested for an *E. coli* cytotoxin (VT) which kills Vero cell cultures (Konowalechuk, Speirs & Stavric, 1977; Scotland *et al.* 1980). Recently, Caprioli *et al.* (1983) described a cytotoxic, necrotizing factor (CNF) in *E. coli* strains isolated from children with diarrhoea. This toxin is a protein (Caprioli *et al.* 1984) that causes necrosis in rabbit skin and, in addition to lethal activity, also causes multinucleation in several types of tissue cultures.

The present paper reports the presence of CNF and other recognized enterotoxins

and cytotoxins, haemolysin and adhesins in *E. coli* strains isolated from children and adults with diarrhoea in northern Italy.

MATERIALS AND METHODS

Population studied

One hundred and sixteen consecutive cases of diarrhoea in children aged from 0 to 6 years and 56 cases in adults admitted to two hospitals and their outpatient clinics in Brescia were examined. Seventy-seven children and 36 adults without symptoms of gastrointestinal disorders were also included in the study. Patients and controls were matched by age and period of admission. Cases of diarrhoea had fluid stools at the time of admission and not less than twice the usual number of stools per day. Further requirements were (i) diarrhoea for at least 24 h, but no longer than 7 days, before admission and (ii) no antibiotic treatment in the preceding week. The study was conducted between January 1980 and March 1981.

Stool examination

Stool specimens were collected at admission and only one specimen from each patient was included in the study. Faeces were examined by conventional techniques for isolation of salmonella, shigella and yersinia (Edwards & Ewing, 1972). Stools were also inoculated on to MacConkey agar, and three lactose-fermenting colonies were picked up and identified as *E. coli* by the API 20E system. For each sample, *E. coli* strains showing different API patterns were studied as described.

Toxin assays

The methods used to detect heat-labile enterotoxin (LT) and cytotoxin production are described in detail elsewhere (Caprioli *et al.* 1983) and summarized here. *E. coli* cultures from sheep blood agar were grown in trypticase soy broth, sonicated and centrifuged; supernates were filter-sterilized and inoculated on to CHO, Vero, HeLa and HEp-2 cells in 96 well microtitre plates. Monolayers were examined during a 4-day period, and the cytotoxic effects of LT (Guerrant *et al.* 1974; Speirs, Stavric & Konowalchuk, 1977) were recorded as well as the death of cells (Konowalchuk, Speirs & Stavric, 1977). Presence of CNF was revealed by multinucleation in at least 50% of cells (Caprioli *et al.* 1983). For heat-stable enterotoxin (ST) detection, bacterial extracts obtained as above were tested in infant mice according to the method of Dean and co-workers (1972).

Haemolysis test

E. coli strains were tested on 5% sheep blood agar plates, and haemolysis halos were scored after overnight incubation at 37 °C.

Test for invasiveness

The *in vitro* technique using HeLa cell cultures described by Mehlman and co-workers (1977) was adopted.

Haemagglutination (HA) pattern determination

HA typing was carried out as described by Evans and co-workers (1980), using erythrocytes from human (type A), guinea pig, bovine, adult chicken and African green monkey. Tests were performed by slide agglutination with bacterial cells grown for 18 h on CFA agar (Evans, Evans & Tjoa, 1977). HA was denoted as mannose resistant (MRHA) if the same degree of HA occurred with and without 1% mannose; mannose sensitive (MSHA) if it was prevented or grossly reduced by the presence of mannose.

Agglutination with anti-colonization factor antigens (CFA) sera

Specific anti-CFA antisera were prepared in rabbits by the procedure of Evans and co-workers (1975), using *E. coli* strains H10407 (CFA/I+) and D1766 (CFA/II+). Crude antisera were absorbed with live cells of strains D1669 (CFA/I-) and D1768 (CFA/II-) until no agglutination was detected. All these strains were a generous gift of Ida and Frits Ørskov, International Escherichia Centre, Copenhagen.

Serotyping

Serotyping was carried out by Ida and Frits Ørskov at the International Escherichia Centre in Copenhagen. O and H antigens were examined by agglutination techniques using the available O and H antisera. Polysaccharide K antigens were examined by immunoprecipitation techniques using the established K antisera (Ørskov & Ørskov, 1978).

Statistical methods. Fisher's exact test and the χ^2 test were used to analyse the incidence of virulence factors in strains of *E. coli* from the different sources (Siegel, 1956).

RESULTS

One hundred and seventy-two patients with diarrhoea and 113 healthy subjects were studied. Neither *Shigella* spp. nor *Yersinia* spp. were isolated, whereas *Salmonella* spp. were found in 47 patients. Two hundred and ninety-nine different strains of *E. coli* were isolated and tested for enterotoxin, cytotoxin and haemolysin production, and haemagglutination and invasive properties. Table 1 shows the results of the assays. Data on *E. coli* strains from patients with and without simultaneous isolation of salmonella are reported separately (groups B and A, respectively). Only three strains of *E. coli* proved enterotoxigenic, none enteroinvasive. Cytotoxin production was revealed in 16 strains: 10 were positive for CNF, 6 for other factors which caused the death of HeLa cells within 4 days. Some of these strains gave less-pronounced cytotoxic effects also on CHO, Vero and HEp-2 cells, which consisted in cessation of growth, vacuolation and, sometimes, in the death of cells; none was proved to produce VT (Table 2).

The frequency of strains producing toxic factors (enterotoxins, cytotoxins, haemolysin) was significantly higher ($P < 0.01$) in the diarrhoea groups A and B (28/185, 15.1%) than in the control group (3/114, 2.6%) (Table 3).

MRHA was found in 25.9% of strains from the patient groups (48/185) and in

Table 1. Virulence factors in strains of *Escherichia coli*, by source

Source	No. of cases	No. of strains	No. of strains with									
			LT	ST	LT+ST	CNF	Hly	CNF+Hly	K*	MRHA	INV†	
Diarrhoea with only <i>E. coli</i> (group A)	125	136	1	1	0	1	7	6	5	36	0	
Salmonella-associated diarrhoea (group B)	47	49	0	0	1	0	3	2	1	12	0	
Healthy subjects (group C)	113	114	0	0	0	0	2	1	0	16	0	

* Toxic factor which kills at least one of the cell lines tested (see Materials and Methods).
 † Invasive property.

Table 2. Effects of bacterial extracts from cytotoxic strains of *Escherichia coli* on different mammalian cell lines

Cell line	Strain									
	B313	A125	A179	A320	A323	A330	E11206/0*	K-12 J53†		
HeLa	+++†	+++	++	+++	+++	+++	+++	-		
CHO	++	++	-	+	-	++	-	-		
Vero	+	-	-	-	-	++	+++	-		
HEp-2	+	+	-	-	-	+	+++	-		

* Strain positive for VT (by B. Rowe, London, UK).
 † Negative control strain.
 ‡ +++ + +, 100% mortality; ++ +, 75% mortality; + +, 50% mortality; +, 25% mortality; -, no effects.

Table 3. *Toxin production and presence of MR adhesins in strains of Escherichia coli, by source*

Source	Strains tested	Strains positive for			
		Toxin*	(%)	MRHA	(%)
Group A†	136	21	(15.4)	36	(26.5)
Group B	49	7	(14.3)	12	(24.5)
Group C	114	3	(2.6)	16	(14.0)

* This includes enterotoxins, cytotoxins, haemolysin. † See Table 1.

Table 4. *Distribution of Escherichia coli strains from different sources, according to HA type*

HA type no.	Strains from					
	Group A*		Group B		No.	(%)
	No.	(%)	No.	(%)		
I	0	—	0	—	0	—
II	0	—	2	(4.1)	0	—
III	12	(8.8)	2	(4.1)	5	(4.5)
IV	45	(33.2)	17	(34.7)	60	(52.6)
V	30	(22.0)	6	(12.3)	12	(10.5)
VI	1	(0.7)	2	(4.1)	3	(2.6)
VII	3	(2.2)	2	(4.1)	0	—
MR†	2	(1.5)	0	—	1	(0.9)
MS‡	43	(31.6)	18	(36.6)	33	(28.9)

* See Table 1. † MRHA patterns not listed in Evans' schema. ‡ MSHA patterns not listed in Evans' schema.

Table 5. *Association between toxin production and presence of MR adhesins in strains of Escherichia coli, by source*

Virulence pattern	Source			
	Group A + B*		Group C	
	185 strains	(%)	114 strains	(%)
Tox††	11	5.9	0	—
MRHA ⁺	31	16.7	13	11.4
Tox ⁺ , MRHA ⁺	17	9.2	3	2.6
Total	59	31.9	16	14.0

* See Table 1. † This includes enterotoxins, cytotoxins and haemolysin production.

14% of strains from controls (16/114); this difference proved significant ($P < 0.05$). The distribution of the strains according to the HA types defined by Evans and co-workers (1980) is shown in Table 4. Most of strains showing MRHA belonged to the HA type V category (49/64, 76.6%); no strain belonged to the HA type I category, characteristic of CFA I positive strains. Two strains fitted in the HA type II; one of them was an ETEC producing both LT and ST and was agglutinated by the CFA/II antiserum. The latter strain, which was isolated from an adult with

Table 6. *Distribution of Escherichia coli serotypes with respect to virulence patterns*

Serotype	No.	Toxin type	Serotype	No.	Toxin type
06:K15:H16	1	LT+ST	01:K5:H1	1	—
043:K15:H10	1	LT	01:K51:H7	1	—
Sp.aggl.:K?:H4*	1	ST	02:K1:H?	1	—
083:K?:H31	1	CNF	02:K?:H4	1	—
02:K7:H1	1	CNF+Hly	03:K2:H2	1	—
06:K2:H1	1	CNF+Hly	07:K16:H6	1	—
013:K12:H16	1	CNF+Hly	08:K28:H—	1	—
018ac:K10:H16	1	CNF+Hly	08:K25:H9	1	—
022:K13:H12	1	CNF+Hly	010:K5:H4	1	—
073:K53:H18	1	CNF+Hly	015:K20:H—	5	—
083:K14:H31	1	CNF+Hly	015:K?:H—	1	—
085:K?:H?	1	CNF+Hly	016:K51:K102:H—	1	—
015:K20:H—	1	CNF+Hly	018ac:K1:H7	1	—
06:K2:H1	2	Hly	020:K—:H—	1	—
015:K14:H4	1	Hly	021:K4:H4	1	—
018ac:K5:H—	1	Hly	021:K?:H21	1	—
018abc:K—:H—	1	Hly	027:K—:H12, H30	1	—
018abc:K—:H—	1	Hly	050:K?:H14	1	—
018abc:K?:H—	1	Hly	051:K?:H49	1	—
021:K2:H2	1	Hly	052:K23, K13:H10	1	—
021:K2:H—	1	Hly	076:K?:H19	1	—
033:K?:H—	1	Hly	078:K—:H—	1	—
083:K14:H31	1	Hly	086:K?:H—	1	—
OX8:K1:H4	1	Hly	086:K?:H10	1	—
02:K1:H7	1	K†	088:K?:H—	1	—
08:K28:H4	1	K	091:K—:H7	1	—
023:K?:H15	1	K	099:K—:H—	1	—
0101:K103:H9	1	K	099:K6:H10	1	—
OX7:K?:H26	1	K	0101:K36:H9	1	—
040:K—:H48	1	K	017,0106:K?:H18	1	—
01:K1:H—	2	—	Rough:K16:K97:H—	1	—
01:K1:H7	1	—	Rough:K?:H—	1	—
01:K—:H19	1	—	0101:K?:H4	1	—

* Spontaneous agglutination. † See Table 1.

salmonella-associated enteritis and was positive for CNF and Hly, failed to react with the antisera to CFA/I and CFA/II. The other two ETEC identified did not show MRHA neither did they react with CFA/I and CFA/II antisera.

The occurrence of toxin production and mannose resistant adhesins and their association in the strains studied are reported in Table 5. Because of their close similarity, the results for group A and B were combined. Virulence factors were found in 31.9% of strains from patients against 14% in the group C ($P < 0.001$). In particular, strains Tox⁺ and strains Tox⁺, MRHA⁺ were significantly more frequent in patient groups than in the control group (5.9% *v.* 0%, $P < 0.001$ and 9.2% *v.* 2.6%, $P < 0.05$, respectively). On the contrary, no significant difference was observed between the two groups in the occurrence of strains showing MRHA alone (16.7% *v.* 11.4%). Overall, 20 strains exhibited both toxin production and

MRHA, accounting for 64.5% (20/31) and 31.2% (20/64) of the toxic strains and MRHA positive strains, respectively.

All *E. coli* strains showing virulence characters were serotyped; the results are shown in Table 6.

DISCUSSION

Strains of *E. coli* isolated from diarrhoeas and healthy subjects have been studied with respect to some virulence traits.

Enterotoxin production was rarely found, thus confirming that sporadic ETEC infections are uncommon in areas of good hygiene and nutrition (Brunton *et al.* 1980; Maki, Vesikari & Grönroos, 1980; Blanco *et al.* 1983); furthermore, the only ST-LT producer strain found, which belonged to the world-wide ETEC serotype 06:H16 (Ørskov *et al.* 1976; Echevarria *et al.* 1982) and possessed CFA/II, had been probably imported to Italy, being isolated from an adult recently arrived from North Africa. An even more minor role seems to be played by enteroinvasive *E. coli*, for we failed to isolate any in the present study. In contrast, 28 strains were found to produce cytotoxic factors or haemolysin. Six of them caused damages in cell cultures, but the factors they produce were shown to be different from VT on the basis of their activity on the cell lines tested; in addition, only faint amounts of toxin were revealed in the extracts of these strains, whereas VT is usually produced at high titre by positive *E. coli* (Konowalchuk, Speirs & Stavric, 1977). Ten further strains were positive for CNF, nine of which proved also haemolytic, thus confirming previous observations of a strong association between these two properties (Caprioli *et al.* 1983). Haemolytic property alone was found in twelve strains.

Altogether, strains producing haemolysin or cytotoxic factors occurred in about 15% of patients against less than 3% of healthy subjects. The involvement of such factors in the pathogenesis of diarrhoea has been suggested (Scotland *et al.* 1980; Caprioli *et al.* 1983; O'Brien & Laveck, 1983), but it still remains unproven. Our data seem to confirm the role of cytotoxins and hemolysin as virulence factors.

Most of toxin-producing strains were also found to possess MR adhesins, as previously described for haemolytic *E. coli* involved in extra-intestinal infections (Evans *et al.* 1981; Hughes *et al.* 1983). This strong association might explain the more frequent observation of MRHA among strains from diarrhoea. In fact, strains either Tox⁺, MRHA⁻ or Tox⁺, MRHA⁺ occurred mostly in patients, whereas the frequency of strains Tox⁻, MRHA⁺ did not differ significantly between cases and controls.

No particular association between serogroups and virulence factors was found; however, most of the strains positive for CNF and/or Hly, belonged to serogroups commonly involved in extra-intestinal infections (Ørskov *et al.* 1977; Evans *et al.* 1981).

In conclusion, our findings indicate that the same aggressive strains of *E. coli* commonly involved in extra-intestinal infections (Evans *et al.* 1981; Hughes *et al.* 1983) might also play a role in diarrhoeal diseases. The occurrence of these strains in the same proportion of patients with or without simultaneous isolation of salmonella, suggests they could act as opportunistic pathogens, colonizing subjects with previous intestinal disorders.

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