Biochemical phenotypes of enteropathogenic *Escherichia coli* common to Iran and Sweden

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Summary. A collection of 143 strains of enteropathogenic *Escherichia coli* (EPEC) of 11 different serogroups isolated from children with diarrhoea, 71 in Sweden and 72 in Iran, was tested for similarity with a computerised biochemical fingerprinting method. From Sweden, there were 54 different phenotypes, 42 consisting of a single strain and 12 (common phenotypes) containing more than one isolate. From Iran, there were 48 different phenotypes, 38 with only one strain and 10 with more than one. Many of the strains which were biochemically similar, in both countries, also had similar virulence factors. Nine Swedish and 20 Iranian isolates showed biochemical identity to at least one of the strains of the other country, most of them belonging to serogroups O55, O119, O126, O127 and O128. The value of the biochemical fingerprinting method as an epidemiological tool and its ability to evaluate clonal relations among *E. coli* strains in different geographical areas is discussed.

Introduction

The term enteropathogenic *Escherichia coli* (EPEC) was first used by Neter¹ for serotypes of *E. coli* that were epidemiologically associated with outbreaks of diarrhoea in infants and young children. This term is currently used to denote serotypes of *E. coli* that cause diarrhoea, but the mechanism by which they elicit the disease is unclear. These strains do not produce enterotoxins and are not invasive by the Sereny test.² Several H types exist within each O group, and it has been shown that strains of each individual serotype, regardless of their geographical origin, not only share O and H antigens but often have common fimbrial antigens and similar outer-membrane-protein (OMP) patterns.³ Furthermore, each such O:H serotype is associated with a specific and often characteristic biotype, making it possible to predict the biotype from the O:H type and *vice versa*, without considering the geographical origin of the isolates.⁴ These findings suggest that each bio/serotype of *E. coli* may represent a clone derived from a common progenitor.⁵

Although O antigen typing of *E. coli* strains can be done easily by testing a boiled suspension of bacteria with commercially available antisera, subdivision of the species for epidemiological studies requires complete serological typing of O, K and H antigens; though this gives good differentiation, it is laborious and time consuming and is performed in only a few laboratories. Biotyping of *E. coli*⁶ with simple tests

has been widely used, alone and in combination with serotyping.^{5,7}

In the present study we used a computerised biochemical fingerprinting method to investigate the presence of common biochemical phenotypes among different serogroups of EPEC strains from two geographically different areas. We also evaluated the presence of three potential virulence factors of EPEC strains within the common phenotypes: EPEC adhesive factor (EAF), verocytotoxin (VT) and haemolysin (HLY).

Materials and methods

Strains of EPEC

A random selection of 143 EPEC strains from children with diarrhoea were tested: 71 had been isolated in Sweden in 1982–1988, and 72 in Iran in 1985–1988. The Swedish strains were obtained from the National Bacteriological Laboratory (NBL), Stockholm; they had been tested for O antigen with polyvalent and monovalent antisera (NBL, Stockholm), and had been stored at –70°C. The Iranian strains were obtained from the collection of the Pasteur Institute of Iran, Tehran; they had been tested for O antigen with Difco polyvalent and monovalent antisera, and had been stored in deep agar at room temperature. K and H antigens had not been determined for either group. The number and distribution of the strains within each serogroup for both countries are given in table I.

Biochemical fingerprinting

A computerised fingerprinting method was used for testing biochemical reactions of the bacteria,^{8,9} by measuring the kinetics of 24 biochemical (fermentation) reactions, chosen to give a high degree of discrimination among *E. coli* strains (D-xylose, maltose, lactose, sorbitol, rhamnose, L-fucose, glycerol, melibiose, deoxyribose, sucrose, raffinose, sorbose, tagatose, D-arabitol, adenitol, dulcitol, ornithine, 5-ketogluconate, melbionate, arbutin, D-arabinose, β -methyl glucoside, lactulose, cellobiose). The results of four readings of these reactions at different time intervals were used to calculate the similarity coefficients between the tested strains. The coefficients were then clustered to yield a dendrogram.

E. coli strains were incubated overnight on MacConkey agar at 37°C, and a single colony was subcultured on nutrient agar. The growth was suspended in 8 ml of a medium containing Proteose Peptone (Difco) 0.1% w/v and bromothymol blue 0.01% w/v as indicator, to give a concentration of $(2-5) \times 10^8$ cells/ml; 150- μ l volumes of these suspensions were inoculated into the wells of the prefabricated microplates containing four sets of 24 dehydrated reagents (Phene Plate System, BioSys Inova, Stockholm), with a multi-channel pipette. The plates were kept at 4°C overnight and incubated at 37°C the next morning. The A_{620} of each reaction was read after 4, 7, 24 and 48 h with a microtitration plate reader (Titertek Multiskan, Flow Laboratories) connected to a microcomputer (Victor-V286C). The similarities between all tested strains were calculated as correlation coefficients (*r*) as described previously,^{8,9} and clustered according to the unweighted pair group method with arithmetic averages (UPGMA).¹⁰

From the reproducibility of the method with 50 strains tested in duplicate, an identity level of 0.975 was chosen (mean $r \pm 2$ SD; 95% confidence level). Strains with *r* values higher than this were regarded as identical, and were assigned to the same biochemical phenotype. Phenotypes with more than one isolate were designated C-phenotypes (common phenotypes), and those with only one isolate were designated S-phenotypes (single phenotypes).¹¹ Capital letters were used for Swedish C-phenotypes, and small letters for Iranian C-phenotypes (table I).

Homogeneities in the bacterial populations were calculated in two ways: $\bar{r}_1$ is the mean of correlation coefficients between all isolates in each O-group from one country, and $\bar{r}_2$ is the mean for all isolates of each O-group from both countries.

Tests for virulence factors

Adhesion. The presence of EAF was assessed by testing the ability of the bacteria to colonise HeLa cells, as described by Scaletsky *et al.*¹² HeLa cells were grown in CO₂ 5% at 37°C in medium 199 with Earle's salts, supplemented with penicillin 100 U/ml, strep-

tomycin 100 μ g/ml, 2 mM glutamine and fetal bovine serum 10%. A 1-ml volume of 10^5 cells/ml in growth medium was placed in 24-well (16 mm diameter) tissue-culture dishes (Flow Laboratories, Irvine, Scotland) covered with sterile 13-mm coverslips. To each well was added 25 μ l of bacterial culture, grown in Tryptic Soy Broth (TSB; Difco) at 37°C overnight; and plates were incubated for 30 min at 37°C in CO₂ 5%. Plates were then washed six times with sterile phosphate-buffered saline pH 7.2 (PBS) and were refilled with the growth medium and incubated for 3 h. Cells were then washed three times with PBS, fixed with methanol, stained with undiluted May-Grünwald stain (5 min) and Giemsa stain 1 in 20 (15 min) and rinsed with PBS. Cover slips were removed to microscope slides, dried in the air and observed by light microscopy. Attachment of the bacteria to HeLa cells as a cluster or microcolony was designated localised adhesion (LA) whereas attachment over the entire surface of the cells was designated diffuse adhesion (DA).¹² To study the possibility of mannose-sensitive adhesion, due to the expression of type 1 (common) pili, all tests were done in duplicate, in the presence and absence of D-mannose 1% in the growth medium.

VT assay. Filtrates of lysed bacteria were prepared by the method of Baloda *et al.*¹³ Confluent Vero cell monolayers were obtained in 96-well microtitration plates incubated for 3 days at 37°C in CO₂ 5%, each well containing 250 μ l of medium 199 with Eagle salts, supplemented with fetal calf serum 10%. The growth medium was substituted with fresh medium containing fetal calf serum 2%, 25 μ l of bacterial culture filtrates were added, and the plates were incubated for 24 h at 37°C in CO₂ 5%. Cells were then examined for cytopathic effect,¹⁴ all tests being done in duplicate. Filtrate of a TSB culture of *E. coli* O157:H7 (VT+, HLY+) was used as positive control; PBS and TSB were used as negative controls.

HLY production. Defibrinated sheep blood was washed three times with 0.01 M Tris chloride buffer, pH 7.5, containing 0.135 M NaCl, and the erythrocytes were added to Blood Agar Base No. 2 (Oxoid) at a final concentration of 5%. Plates of this medium were inoculated with EPEC and examined for haemolysis after overnight incubation at 37°C.

Results

Swedish isolates

Among the 71 Swedish isolates, 12 common (C) phenotypes, containing 2–4 strains each, and 42 single (S) phenotypes were identified. Strains of C-phenotypes belonged to serogroups O26, O44, O55, O111, O114, O119, O125, O126 and O127 (table I). More than one biochemical phenotype were observed within serogroups O44, O55 and O126. The two C-phenotypes in group O44 (phenotypes B₁ and B₂) were closely

Table I. Common biochemical phenotypes (C-phenotypes) and single strains (S-phenotypes) among 71 Swedish and 72 Iranian isolates of EPEC

Serogroup	Sweden				Iran				Total	
	Number of strains	C-phenotypes* (number of strains)	Number of S-phenotypes	$\bar{r}_1$ †	Number of strains	C-phenotypes* (number of strains)	Number of S-phenotypes	$\bar{r}_1$ †	Number of strains	$\bar{r}_2$ †
O26	10	A(4)	6	0.785	3	...	3	0.579	13	0.705
O44	7	B ₁ (2) B ₂ (2)	3	0.837	2	...	2	0.934	9	0.781
O55	5	C(2) D(2)	1	0.556	4	a(3) j(1)	0	0.967	9	0.649
O86	6	...	6	0.735	5	b(3) i(1)	1	0.752	11	0.722
O111	8	E(2)	6	0.755	8	c(2) d(2)	4	0.732	16	0.750
O114	9	F(4)	5	0.757	4	...	4	0.696	13	0.713
O119	6	G(2)	4	0.841	4	...	4	0.620	10	0.737
O125	6	H(3)	3	0.652	3	...	3	0.665	9	0.597
O126	6	I(2) J(2)	2	0.808	16	e(4) f(2)	10	0.670	22	0.688
O127	5	K(2)	3	0.696	5	g(4)	1	0.949	10	0.738
O128	3	...	3	0.694	18	h(2) i(4) g(6)	6	0.822	21	0.794

* C-phenotypes are given capital letters for Swedish isolates and small letters for Iranian isolates.

† $\bar{r}_1$ indicates the homogeneity (mean of correlation coefficients) for the isolates of each serogroup from one country; $\bar{r}_2$ for both countries.

related to each other (similarity level 0.965): phenotype B₂ had slower rates of fermentation of dulcitol, D-arabinose and lactulose than those of B₁. The C-phenotypes within groups O55 (phenotypes C and D) and O126 (phenotypes I and J) were unrelated. Duplicate assays of VT production by the Swedish isolates showed no discrepancies: seven strains were VT producers, six of them being of C-phenotype A, D or E (table II). Mannose-sensitive adhesion (MSA) to HeLa cells was shown by 34 strains (48%): LA with 17 strains, DA with 14 strains, both patterns (LA/DA) with three strains. Of these, 33 strains also showed mannose-resistant adhesion (MRA) and 20 of them belonged to 10 C-phenotypes (table II). Only seven strains produced HLY, and all of them belonged to C-phenotype A, B₂ or D (table II).

Iranian isolates

Among the 72 Iranian isolates, 10 C-phenotypes, containing 2–10 strains each, and 38 S-phenotypes were identified. Strains of C-phenotypes belonged to serogroups O55, O86, O111, O126, O127 and O128 (table I), some serogroups containing more than one phenotype, e.g., serogroups O111 (phenotypes c and d), O126 (phenotypes e and f) and O128 (phenotypes h, i and g). C-phenotype g also contained four strains

of group O127; and two isolates belonging to groups O55 and O86 were both of C-phenotype j (table I). Five VT producers and four HLY producers were of C-phenotype a, c, d or g (table III). MSA to HeLa cells was shown by 30 strains (42%): LA with 20 strains, DA with nine strains, LA/DA with one strain. Of these, 28 strains also showed MRA and 21 belonged to nine C-phenotypes (table III).

Comparison of Swedish and Iranian isolates

The total of 143 isolates could be allocated to 96 different phenotypes, of which 20 were common phenotypes consisting of 2–13 isolates each, and the other 76 were single isolates. The figure shows a dendrogram derived from an UPGMA clustering of the 20 common phenotypes.

Nine Swedish isolates (three C-phenotypes and three single isolates) and 20 Iranian isolates (four C-phenotypes and one single isolate) showed identity to at least one of the isolates of the other country. Most of these strains were of serogroup O55, O119, O126, O127 or O128 (figure). Two Iranian strains of groups O55 and O86 (C-phenotype j), and one Swedish strain of group O126 were identical in biochemical phenotype. The most common phenotype in the whole material contained 13 isolates: two Swedish strains of

Table II. Virulence factors of common biochemical phenotypes (C-phenotypes) among 71 strains of EPEC from Sweden

Strain no.	Year of isolation	Serogroup	C-phenotype	MRA HeLa cell adhesion	Verotoxin production	Haemolysin production
C154	1987	O26	A	—	+	+
C140	1988			—	+	+
C1	1988			—	+	+
C125	1988	O44	B1	—	+	—
C144	1987			DA	—	—
E199c	1982			—	—	—
C139	1987	O44	B2	LA	—	+
C146	1986			LA	—	+
C183	1988	O55	C	LA	—	—
C100	1987			LA	—	—
C135	1987	O55	D	—	+	+
C124	1987			—	—	+
C106	1987	O111	E	LA	—	—
C113	1987			LA	+	—
C48	1987	O114	F	LA	—	—
RS381B	1983			LA	—	—
RS424S	1983			LA	—	—
C606	1987	O119	G	LA	—	—
C279	1986			LA/DA	—	—
C6	1987			—	—	—
C120	1987	O125	H	—	—	—
C156	1986			LA	—	—
C190	1988			LA	—	—
C220	1985	O126	I	LA	—	—
C241	1985			LA	—	—
C274	1984	O126	J	LA	—	—
C178	1988			LA	—	—
C199	1986	O127	K	LA/DA	—	—
C197	1986			LA/DA	—	—

LA, localised adhesion; DA, diffuse adhesion; LA/DA, both types.

Table III. Virulence factors of common biochemical phenotypes (C-phenotypes) among 72 strains of EPEC from Iran

Strain no.	Year of isolation	Serogroup	C-phenotype	MRA HeLa cell adhesion	Verotoxin production	Haemolysin production
57	1985	O55	a	LA	—	—
1822	1987			LA	—	—
6678	1987			—	+	+
2125	1985	O86	b	—	—	—
2249	1987			DA	—	—
3845	1988			LA	—	—
2530	1987	O111	c	—	+	—
3542	1987			—	+	+
1114	1987			LA	—	+
2216	1987	O111	d	—	+	+
3441	1985			LA	—	—
3643	1985			LA	—	—
4958	1988	O126	e	LA	—	—
5160	1986			—	—	—
4033	1985			LA	—	—
5143	1985	O126	f	LA	—	—
1317	1985			LA	—	—
1923	1985			LA	—	—
2835	1985	O127	g	LA	—	—
1915	1988			—	+	—
4655	1986			—	—	—
5564	1988	O128	h	LA	—	—
1620	1985			—	—	—
5362	1985			—	—	—
6172	1985	O128	i	LA	—	—
6779	1987			—	—	—
2328	1988			LA	—	—
5666	1987	O128	g	—	—	—
3744	1988			—	—	—
4553	1985			LA/DA	—	—
6467	1986	O128		LA	—	—
2024	1986			LA	—	—
2093	1986			LA	—	—
4173	1987	O55	j	LA	—	—
		O86		LA	—	—

LA, localised adhesion; DA, diffuse adhesion; LA/DA, both types.

C-phenotype K (group O127), 10 Iranian strains of C-phenotype g (groups O127 and O128) and one Swedish S-phenotype of group O128 (figure).

Discussion

Different typing systems have been used to investigate the clonal relations between bacteria in epidemiological studies.¹⁵⁻¹⁷ Major problems with most systems are that all the strains are not typable with one system, or the system is complex and laborious and cannot be used in many laboratories. Furthermore, most of these methods are based only on the establishment of identity or non-identity of the tested strains, and cannot detect possible relations between the isolates. With computerised biochemical fingerprinting, we detected 54 phenotypes among 71 randomly selected EPEC strains from Sweden, and also 48 phenotypes among 72 presumably unrelated strains from Iran. Most strains within each C-phenotype, in both countries, carried the same O antigen and some of them had similar virulence properties.

The presence of a large number of biochemical phenotypes among Swedish and Iranian isolates (54 and 48, respectively) is not surprising, because biochemical reactions among *E. coli* strains vary widely. However, biochemical identity in this study was set at a similarity level of 0.975, based on the reproducibility of the system. Therefore, strains that were biochemically very similar to each other, but fell below this identity level, were assigned to different phenotypes. Moreover, some of the strains which were closely related to C-phenotypes, carried the same O antigen and had similar virulence factors; for instance, the four strains of group O26 belonging to C-phenotype A were very similar to another Swedish strain of the same O group (similarity 0.965; data not shown) that also produced VT but not HLY. Similarly, with four strains of serogroup O44 (phenotypes B₁ and B₂), another closely related strain not only belonged to the same O group but also showed LA.

Bacterial clones which exist for a long time in a given population may undergo changes in phenotypic characters,⁴ and lose virulence factors.¹¹ We have reported previously that the biochemical phenotypes of EPEC strains may be more stable on subculture

Figure. Dendrogram of 32 Swedish (---) and 35 Iranian (—) strains of EPEC. Common biochemical phenotypes (C-phenotypes) are shown with capital letters for Swedish and small letters for Iranian strains. The discontinuous vertical line ($r=0.975$) indicates the chosen identity level for strains to be assigned to the same biochemical phenotype. This two-dimensional model produces some discrepancies: for example, the two Swedish O127 isolates (K) were phenotypically different from the single O128 isolate ($r<0.975$), but all three showed phenotypic identity ($r>0.975$) with the similar cluster (g) from Iran.

and storage than those of normal faecal *E. coli* strains.¹⁸ In contrast, most of the virulence factors of *E. coli* strains may be lost after such treatment.¹⁹⁻²¹ Therefore, it is possible that some strains of one biochemical phenotype, including closely related strains, might have lost some virulence factors during storage and would have appeared as not identical if only their virulence factors were evaluated. In our study, EAF and HLY were measured only once; this may explain small differences in virulence factors among the strains of each C-phenotype, as some variation in duplicate assays is to be expected.

Among Iranian isolates, 10 C-phenotypes, belong-

ing to six different O groups, were observed. Twelve strains of group O128 belonged to three different phenotypes (h, i and g; see table III). This serogroup has been shown previously to account for the majority of the EPEC strains isolated from diarrhoeal cases in Iran.^{22, 23} Our present results show that three major and probably related phenotypes ($\bar{r}_1=0.822$) in this serogroup are particularly common in Iran, and infection by this serogroup is thus probably due to the spread of three clones. We also found one Swedish strain carrying this O antigen and belonging to biochemical phenotype g (see figure).

Four Iranian strains of group O127, also, were of

the same biochemical phenotype (type g) as six strains of group O128, showing that strains of two different serogroups might be biochemically related. The clone concept has been defined as "bacterial cultures isolated independently from different sources, at different geographical areas and at different times, but showing so many identical phenotypes and genetic traits that the most likely explanation for this identity is a common origin".⁴ Nine Swedish and 20 Iranian isolates in this study showed biochemical identity to at least one of the strains of the other country and most of them (26 strains) also carried the same O antigen. Furthermore, many of the strains within each C-phenotype had similar virulence factors (EAF, VT, HLY). In an analysis of OMP patterns of 129 strains of *E. coli* belonging to different serotypes of groups O55 and O111, isolated from geographically distinct areas, Stenderup and Ørskov⁵ found stable sero/bio/OMP types, and concluded that such highly defined phenotypes represent bacterial clones that have been spread worldwide. The presence of common serogroups and phenotypes among the EPEC strains in our study confirms those results and the theories of widespread pathogenic clones.

Kühn⁸ showed that, with large numbers of randomly selected isolates of *E. coli*, mean homogeneities for biochemical phenotypes varied between 0.62 and 0.66. If the homogeneity is higher, this indicates that some of the isolates may be related. In the present study, we found a high homogeneity among the strains of most O groups. However, five Swedish isolates of group O55 (two C-phenotypes and one S-phenotype) and three Iranian isolates of group O26 (three S-phenotypes) had very low homogeneity (0.556 and 0.579 respectively); this indicates that they belonged to totally unrelated clones which have spread in these countries.

Three strains (Iranian C-phenotype j and one Swedish isolate) were found to belong to the same biochemical phenotype, but had three different O antigens. The reason is unclear. Because these strains had been tested originally with different sets of antisera, we re-tested them with both sets and obtained similar results.

Serotyping of *E. coli* strains has been considered to reveal uniform genetic entities and, therefore, meaningful units of the population structure.^{24, 25} However, this view has been questioned. For instance, in an analysis of chromosomal-gene diversity by multilocus enzyme electrophoresis of 261 strains of *E. coli* with various O, K and H antigens, Gaugant *et al.*²⁶ found that most strains of the same electrophoretic type (ET) shared the same combination of antigens. However, some antigenic diversity was also observed among isolates of the same ET. Our results of biochemical fingerprinting of EPEC strains from Sweden and Iran showed that most of the isolates of the same biochemical phenotype carried the same O antigen. However, like the above workers, we also found that certain biochemical phenotypes (e.g., Iranian C-phenotype g) included isolates of different serogroups (O127 and O128). Nevertheless, the fact that some strains of each C-phenotype, within each serogroup, also had similar virulence factors supports the previous findings by Kühn *et al.*¹¹ and Kühn and Möllby²⁷ that the biochemical fingerprinting method described above, together with O antigen typing, could be an easy and useful method in identifying *E. coli* clones of diverse origin.

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