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Serotype-related enterotoxigenicity in *Escherichia coli* O6.H16 and O148.H28

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SUMMARY

The ability of certain *Escherichia coli* strains to produce enterotoxin is determined by transmissible plasmids. It is therefore possible that any *E. coli* strain might be able to acquire such a plasmid and that the correlation between enterotoxigenicity and serotype might be random. However, recent studies show that the enterotoxigenic strains so far described belong to a restricted range of serotypes. Enterotoxigenic strains of *E. coli* O6.H16 and *E. coli* O148.H28 have been associated with outbreaks of diarrhoea in several countries, therefore strains of *E. coli* belonging to these serotypes were selected for further study.

Twenty-three strains of *E. coli* O6.H16 and 14 strains of *E. coli* O148.H28 were examined; 20 strains of *E. coli* O6.H16 and all 14 strains of *E. coli* O148.H28 were enterotoxigenic but strains of *E. coli* O6 with flagellar antigens other than H16 and strains of *E. coli* O148 with flagellar antigens other than H28 were not enterotoxigenic. The examination of single colony subcultures derived from the *E. coli* O6.H16 strains showed that in some strains loss of enterotoxigenicity had occurred in a proportion of colonies.

INTRODUCTION

During the last few years it has been shown that enterotoxigenic *Escherichia coli* (ETEC) are an important cause of diarrhoeal disease in adults and infants, particularly in tropical areas and areas of poor hygiene (Sack, 1975). The ability to produce enterotoxin has been shown in some ETEC strains to be controlled by a transmissible plasmid (Gyles, So & Falkow, 1974). It is therefore possible that an *E. coli* strain of any serotype might be able to acquire such a plasmid and that the association between enterotoxigenicity and serotype might be random. However, Ørskov *et al.* (1976) have reported that ETEC may belong to a relatively small number of serotypes although these are not the same as the traditional infantile enteropathogenic *E. coli* (EPEC) serotypes.

Since the routine investigation of *E. coli* enteritis depends heavily upon the use of serotyping it is important to investigate further the relation between enterotoxigenicity and serotype. Enterotoxigenic *E. coli* belonging to O:H sero-

types O6.H16 and O148.H28 have been reported in epidemics in several countries (Rowe, Scotland & Gross, 1977), therefore cultures of *E. coli* belonging to these serotypes were selected for further study.

MATERIALS AND METHODS

Organisms

All the organisms were from the culture collection of the Salmonella and Shigella Reference Laboratory (SSRL). They had been selected as single colony subcultures from blood agar plates shortly after isolation and maintained thereafter on Dorset egg medium at room temperature.

Twenty-three cultures of *E. coli* O6.H16 were examined and their sources and dates of isolation are shown in Table 1. These represented all the cultures of this serotype received at the SSRL since 1965 and included cultures from an outbreak of infantile enteritis in Gloucester, England (Rowe *et al.* 1977), and from an outbreak of diarrhoea in hospital staff in Worcester, England. For comparison 20 strains belonging to O group O6 but with flagellar antigens other than H16 were also tested for enterotoxin production; of these 4 were non-motile, 9 had flagellar antigen H1 and 7 had H31. These were the most common H-types of *E. coli* O6 received at the SSRL; they were all isolated during the period 1976–7 from faecal specimens of patients with diarrhoea.

Fourteen cultures of *E. coli* O148.H28 were examined although 10 of these were isolated during a single outbreak of diarrhoea in Aden (Rowe, Taylor & Bettelheim, 1970) and one was strain B7a which was originally isolated from an adult with diarrhoea in Vietnam (DuPont *et al.* 1971) and has been widely used as an enterotoxigenic control strain. These 14 cultures included all the epidemiologically unrelated strains of this serotype received at the SSRL since 1965. Further cultures from the Aden outbreak were available but were not included in this study. The sources of the *E. coli* O148.H28 cultures and their dates of isolation are shown in Table 2. For comparison 37 strains belonging to O group O148 but with flagellar antigens other than H28 were also included; of these 11 were non-motile, 15 had flagellar antigen H53, 10 had H30 and 1 had H8. They were all isolated during the period 1965–77 from faecal specimens of patients with diarrhoea. No other H types of *E. coli* O148 were received at SSRL during this period.

Enterotoxin tests

The organisms were tested for the production of heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT). The infant mouse test of Dean, Ching, Williams & Harden (1972) was used for the detection of ST while the Y1 adrenal cell test (Donta, Moon & Whipp, 1974) and the CHO cell test (Guerrant *et al.* 1974) were used to detect LT. The enterotoxin preparations were inoculated direct from the Dorset egg cultures in the culture collection.

Since some of the *E. coli* O6.H16 cultures were non-enterotoxigenic when tested in this way, the loss of enterotoxigenicity in these cultures was examined by testing a number of single colony subcultures. All the cultures of this serotype

Table 1. *Cultures of* E. coli *O6.H16*

Culture no.	Place of isolation	Year of isolation	Source of culture	Enterotoxin production using sweep inoculum: LT	Enterotoxin production using sweep inoculum: ST	No. LT+ single colonies	ST production by LT+ colony	Drug resistance†	Colicin production†	Biotype†
E23/66	Venezuela	1966	Bird	−	−	0/100	No LT+ found	Fu, Sm, Su, Tc	+	E
E1052/66	Aden	1966	British soldier with diarrhoea	−	−	2/100	+	Su	−	A
E1069/66	Aden	1966	British soldier with diarrhoea	+	+	1/10	+	Su	−	A
E201/69	Sharjah	1969	British soldier with diarrhoea	+	+	5/10	+	None	−	C
E219/69	Sharjah	1969	British soldier with diarrhoea	+	+	8/10	+	None	−	C
E1437/69	Sharjah	1969	British soldier with diarrhoea	−	−	0/100	No LT+ found	None	−	F
E2047/69	U.K.	1969	Infant with diarrhoea	+	+	2/10	+	None	−	B
E1539/70	U.K.	1970	Infant with diarrhoea	−	−	0/100	No LT+ found	Su	+	G
E1064/72	Ethiopia	1972	Adult with diarrhoea	+	+	10/10	+	None	−	C
E651/73	Worcester, U.K.	1973	Outbreak of diarrhoea among hospital staff	−	−	2/100	+	None	−	A
E652/73	Worcester, U.K.	1973	Outbreak of diarrhoea among hospital staff	−	−	3/100	+	None	−	A
E653/73	Worcester, U.K.	1973	Outbreak of diarrhoea among hospital staff	+	−	2/10	+	None	−	A
E1392/75	Hong Kong	1975	Adult with diarrhoea	+	+	8/10	+	Sm, Su	−	A
E2337/75	Hong Kong	1975	Adult with diarrhoea	+	−	9/10	−	None	−	B
E3482/76	Canada*	1975	Infant with diarrhoea	+	+		Not tested	None	+	B
E4683/76	Gloucester, U.K.	1976	Outbreak of diarrhoea in special care baby unit	+	+		Not tested	None	−	C
E4688/76	Gloucester, U.K.	1976	Outbreak of diarrhoea in special care baby unit	+	+		Not tested	None	−	C
E4728/76	Gloucester, U.K.	1976	Outbreak of diarrhoea in special care baby unit	+	+		Not tested	None	−	C
E4733/76	Gloucester, U.K.	1976	Outbreak of diarrhoea in special care baby unit	+	+		Not tested	None	−	C
E4753/76	Gloucester, U.K.	1976	Outbreak of diarrhoea in special care baby unit	+	+		Not tested	None	−	C
E5470/76	Gloucester, U.K.	1976	Outbreak of diarrhoea in special care baby unit	+	+		Not tested	None	−	C
E4833/76	Canada*	1976	Infant with diarrhoea	+	+		Not tested	None	+	B
E4904/76	Canada*	1976	Infant with diarrhoea	+	+		Not tested	None	+	D

* Received from Dr M. Gurwith.

† Where LT+ and LT− colonies were found, both were tested and gave identical results.

Table 2. *Cultures of* E. coli *O*148.*H*28

Culture no.	Place of isolation	Year of isolation	Source of cultures	Enterotoxin production	
				LT	ST
E517/66	Aden	1965	Outbreak of diarrhoea among British troops	−	+
E519/66				−	+
E523/66				−	+
E525/66				−	+
E540/66				−	+
E590/66				−	+
E596/66				−	+
E600/66				−	+
E1012/66				−	+
E1013/66				−	+
E2289/72*	Japan	1972	Community outbreak of diarrhoea	−	+
E1699/73	Dubai	1973	Infant; no diarrhoea	−	+
E1394/75	U.K.	1975	Adult with diarrhoea on return from Far East	−	+
B7a†	Vietnam	Not known	U.S. soldier with diarrhoea	+	+

* Received from Dr R. Sakazaki.
† Received from Dr S. Formal.

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which had been received at SSRL more than one year before the present study or which failed to produce detectable ST when first tested were subcultured onto blood agar and colonies selected for further examination. At first 10 colonies were selected and tested individually for LT production using the Y1 tissue miniculture assay described by Sack & Sack (1975). If no enterotoxigenic colonies were found a further 100 colonies were selected and mixed in pools of 10; 10 pools of 10 colonies each were then tested for LT production in the Y1 tissue miniculture assay. If any pool gave positive results the 10 individual colonies included in the pool were then tested individually. Any single colony subcultures found to produce LT by this method were then re-tested using the CHO cell test and were tested for ST production using the infant mouse test.

Biotyping

All the cultures of *E. coli* O6.H16 and O148.H28 were tested for their ability to utilize mucate and to ferment glucose, lactose, mannitol, sucrose, salicin, dulcitol, inositol, adonitol, raffinose, sorbitol, arabinose, rhamnose, xylose, trehalose, inulin, glycerol, cellobiose, sorbose and maltose. If enterotoxigenic and non-enterotoxigenic variants were isolated from a single culture then single colony subcultures of each variant were tested.

Drug sensitivity tests

The cultures and single colony subcultures which were biotyped were also tested for sensitivity to a range of antibacterial agents using an agar plate dilution technique (Haltalin, Markley & Woodman, 1973). Each organism was tested for sensitivity to the following agents: ampicillin (10, 25 and 500 μg/ml), cephaloridine (30 μg/ml), chloramphenicol (10 and 30 μg/ml), gentamicin (10 and 30 μg/ml), nalidixic acid (30 μg/ml), neomycin (10 and 30 μg/ml), nitrofurantoin (30 and 200 μg/ml), polymixin B (100 and 300 units/ml), streptomycin (10, 30 and 800 μg/ml), sulphathiazole (100 and 250 μg/ml), tetracycline (10 and 30 μg/ml), trimethoprim (1·25 μg/ml).

Colicin production

The cultures and single colony subcultures which were biotyped were also tested for colicin production using the method of Fredericq (1957).

RESULTS

Enterotoxin testing

Using enterotoxin preparations inoculated directly from the stored cultures 15 of the 23 *E. coli* O6.H16 cultures were shown to produce ST and LT while a further 2 appeared to produce LT only (Table 1). All the 14 *E. coli* O148.H28 cultures were enterotoxigenic; 13 were found to produce ST only and one (strain B7a) produced ST and LT (Table 2). None of the cultures of *E. coli* O6 with flagellar antigens other than H16 nor any of the cultures of *E. coli* O148 with flagellar antigens other than H28 were found to be enterotoxigenic in any of the tests.

Table 3. *Serotype-related enterotoxigenicity*

Serotype	Strains studied	Entero-toxigenic	ST only	LT only	ST plus LT
O6.H16	23	20	0	1	19
O6.H (various)	20	0	0	0	0
O148.H28	14	14	13	0	1*
O148.H (various)	37	0	0	0	0

* = Strain B7a.
Various H types of O6 were H1, H31, H–.
Various H types of O148 were H8, H30, H53, H–.

Table 4. *Biochemical reactions of* E. coli *O6.H16*

Biotype	Utilization of mucate	Fermentation of				
		Adonitol	Dulcitol	Raffinose	Rhamnose	Sorbose
A	+	+	−	−	−	−
B	+	+	−	−	+	−
C	+	+	−	+	+	−
D	+	+	+	−	+	+
E	−	−	−	−	+	−
F	+	−	−	−	+	−
G	−	+	−	−	+	−

The 8 cultures of *E. coli* O6.H16 which failed to produce detectable ST using preparations inoculated directly from the stored cultures were examined further by testing single colony subcultures for LT production. In this way LT producing colonies were found in 5 of these 8 cultures and the LT producing colonies from 4 of these 5 also produced ST. By adding the results obtained by all methods, a total of 20/23 cultures of *E. coli* O6.H16 were found to produce LT and 19 of these also produced ST. The remaining 3 cultures contained less than 1% LT producing colonies (Table 3).

The proportion of LT producing colonies found in the *E. coli* O6.H16 cultures which were more than 1 year old ranged from 2 out of 100 to 10 out of 10. The loss of enterotoxigenicity was not consistently related to the age of the cultures.

Biotyping

All the *E. coli* O6.H16 cultures fermented glucose, lactose, mannitol, maltose, salicin, sorbitol, arabinose, xylose, trehalose and glycerol; they failed to ferment inositol, inulin, cellobiose and sucrose. On the basis of their reactions in the six remaining tests, seven biotypes were recognized (Table 4). When the cultures were from outbreaks of enteritis all the cultures from the same outbreak belonged to the same biotype. When enterotoxigenic and non-enterotoxigenic variants were isolated from the same culture, they invariably belonged to the same biotype. No single reaction or pattern of reactions was consistently related to the presence or absence of enterotoxigenicity.

All the *E. coli* O148.H28 cultures utilized sodium mucate and fermented glucose, lactose, mannitol, sucrose, salicin, raffinose, sorbitol, arabinose, rhamnose,

xylose, trehalose, glycerol and maltose; they failed to ferment inositol, adonitol, inulin, cellobiose and maltose; only two cultures (B7a and E2289/72) fermented dulcitol. There were no other differences of biotype found.

Drug sensitivity tests

Eighteen of the 23 cultures of *E. coli* O6.H16 were sensitive to all the reagents tested. Three cultures were resistant to sulphathiazole, one to sulphathiazole and streptomycin and one to tetracycline, nitrofurantoin, streptomycin and sulphathiazole (Table 1). When enterotoxigenic and non-enterotoxigenic variants were isolated from the same culture, they invariably had the same sensitivity pattern. No resistance pattern was consistently related to the presence or absence of enterotoxigenicity.

Among the cultures of *E. coli* O148.H28 only two cultures were resistant to any of the reagents tested. Strain number B7a was resistant to chloramphenicol, sulphathiazole and streptomycin and strain number E1699/73 was resistant to sulphathiazole and streptomycin.

Colicin production

Five cultures of *E. coli* O6.H16 were found to be colicinogenic; three of these were enterotoxigenic strains isolated in Canada and the other two were non-enterotoxigenic. None of the *E. coli* O148.H28 were colicinogenic.

DISCUSSION

The demonstration that the genetic determinants of enterotoxin production may be carried by transmissible plasmids leads to the possibility that the association between serotype and enterotoxigenicity might be random. However, Ørskov and his colleagues (1976) have recently shown that the ETEC so far described fall into a relatively small number of serotypes. Enterotoxigenic *E. coli* O6.H16 have been reported as a cause of outbreaks of diarrhoeal disease in the United States (*Morbidity and Mortality*, 1975), Mexico (Merson *et al.* 1976), Japan (Kudoh *et al.* 1977) and the United Kingdom (Rowe *et al.* 1977). Similarly enterotoxigenic *E. coli* O148.H28 have been reported in Aden (Rowe *et al.* 1970), Japan (R. Sakazaki, pers. comm.) and Vietnam (DuPont *et al.* 1971). In the present study it has been shown that a high proportion of strains belonging to these serotypes are enterotoxigenic even though the strains were selected only because of their serotype. Ørskov and his colleagues (1976) have stressed that it is the O:H serotype which appears to be associated with enterotoxigenicity and not simply the O antigen. Our findings support this observation since strains of *E. coli* O6 with flagellar antigens other than H16 and strains of *E. coli* O148 with flagellar antigens other than H28 were all non-enterotoxigenic.

It has been suggested that the ability to produce enterotoxin might be unstable and that strains of *E. coli* might lose this ability during storage (Sack, 1976). Our studies confirm that loss of enterotoxigenicity occurs in some stored cultures

but this loss does not occur rapidly and is not consistently related to the age of the cultures or to their biotype, antibiotic resistance pattern or colicinogeny. The *E. coli* O148.H28 cultures all remained enterotoxigenic although some of them had been stored on Dorset egg medium at room temperature since 1965. However, single colony subcultures were not studied and the cultures may have contained non-enterotoxigenic variants. Loss of enterotoxigenicity had occurred in some of the *E. coli* O6.H16 cultures so that in some cultures only 2 out of 100 colonies were found to be enterotoxigenic, while in other cultures, sometimes of greater age, up to 10 out of 10 colonies remained enterotoxigenic.

Enterotoxin testing by the currently available methods is a laborious process unsuitable for use in the clinical laboratory. Testing for ST is a particularly time-consuming process and requires an animal model, nevertheless strains producing ST alone are important in diarrhoea of adults (Kudoh *et al.* 1977) and in infantile enteritis (Ryder *et al.* 1976; Gross *et al.* 1976) and these strains cannot be ignored. Serotyping, on the other hand, is a relatively simple process and most clinical laboratories are familiar with tests for O antigen identification. The demonstration that enterotoxigenicity may be largely restricted to members of a few serotypes offers the possibility that serotyping might be used for the provisional identification of strains which may be enterotoxigenic; such strains could then be tested for enterotoxigenicity in a central laboratory. Further studies are required to determine whether identification of O group alone may be of value in the routine investigation of infantile enteritis due to ETEC or whether determination of O:H serotype is necessary.

There are several possible explanations for the association between enterotoxin production and serotype. Strains of certain serotypes may have a particular ability to acquire enterotoxin plasmids or may, having acquired such a plasmid, retain it more readily. Alternatively the strains which we have studied may represent single clones of *E. coli* which acquired an enterotoxin plasmid at a single point in time and which have since spread to many countries. The observation that enterotoxigenic *E. coli* O6.H16 strains belong to a variety of biotypes argues against the possibility that these strains belong to a single clone but does not exclude this possibility; new biotypes may have arisen within the clone after the acquisition of the enterotoxin plasmid. In order to determine which of these explanations is the most likely, genetic transfer studies are required to establish the frequency of acquisition of enterotoxin plasmids by strains of different serotypes and to determine the stability of the plasmids once they have been acquired.

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