

## Strains of *Escherichia coli* 0157:H8 from human diarrhoea belong to attaching and effacing class of *E coli*

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### Abstract

**Aims:** To determine whether 17 *Escherichia coli* 0157:H8 strains isolated from patients with diarrhoea in the United Kingdom were putative pathogens.

**Methods:** The strains had been isolated by the use of 0157 antiserum, available for the detection of Vero cytotoxin (VT) producing strains of *E coli* 0157 that are usually of flagellar (H) type 7, but may also be non-motile. The strains were examined for VT production, for their ability to adhere to HEp-2 cells, and for hybridisation with several DNA probes that recognise pathogenic properties of *E coli*. Their ability to ferment sorbitol and to produce  $\beta$ -glucuronidase was also investigated, as these tests are used to discriminate VT positive 0157 strains.

**Results:** The 0157:H8 strains did not produce VT. All gave localised attachment to HEp-2 cells, associated with a positive fluorescence-actin staining test, and all hybridised with the *E coli* attaching and effacing (*eae*) probe. In addition to the difference in VT production, 0157:H8 strains could be distinguished from VT positive 0157 strains by their  $\beta$ -glucuronidase activity, their failure to produce enterohaemolysin, and their lack of hybridisation with the CVD419 probe derived from a plasmid in an 0157:H7 strain.

**Conclusions:** The 0157:H8 strains had in vitro properties characteristic of the class of *E coli* that causes attaching and effacing lesions in epithelial intestinal cells. They may therefore be considered a putative cause of diarrhoea but their prevalence remains to be established. Several 0157:H8 strains failed to ferment sorbitol in agar plates and therefore could be misidentified as VT positive 0157 strains. Confirmatory tests for VT production are needed when 0157 strains are isolated from faeces.

haemorrhagic colitis and haemolytic uraemic syndrome in North America and the United Kingdom.<sup>3</sup> These strains produce one or more Vero cytotoxins (VT) and carry a plasmid of molecular size about 60 megadaltons; such strains have been termed enterohaemorrhagic *E coli* (EHEC).<sup>4</sup> The VT positive strains of *E coli* 0157 are usually of H type 7, but non-motile strains have also been reported. Unlike most strains of *E coli*, most VT positive strains of serogroup 0157 do not ferment sorbitol within one day.<sup>5</sup> Sorbitol MacConkey agar (SMAC agar) which contains sorbitol in place of lactose makes use of this characteristic and may be used for faecal culture and the recognition of these organisms.<sup>6</sup> Pale colonies after overnight incubation must be confirmed biochemically as *E coli*, and antiserum, usually in the form of latex agglutination kits, is used to confirm that they are serogroup 0157.<sup>7</sup> Notwithstanding this, there have been reports of atypical sorbitol fermenting VT positive 0157 strains from Germany and the United States.<sup>8,9</sup>

The Division of Enteric Pathogens, in its role as a national reference facility, has received *E coli* 0157 strains isolated from patients for the confirmation of VT production and determination of VT type and phage type for epidemiological purposes. Most of these strains were indeed VT positive 0157 strains, but a small number of VT negative 0157 strains were received and most of these were H type 8. Because the 0157:H8 strains were isolated from patients with intestinal disease we examined them for several relevant pathogenic properties. These included tests for attachment to cells grown in culture. In some enteropathogenic *E coli* (EPEC) the ability to cause clinically important diarrhoea in volunteer studies and good attachment in vitro is dependent on plasmid encoded genes. The 0157:H8 strains were tested for hybridisation with the EPEC adherence factor (EAF) probe developed from one such plasmid.<sup>10</sup> The fluorescence-actin staining (FAS) test<sup>11</sup> which correlates with the ability to cause attaching and effacing (AE) lesions of the intestinal microvilli, was performed and also hybridisation with the *eae* probe derived from chromosomal sequences necessary for the ability to cause AE lesions.<sup>12</sup>

### Methods

*E coli* 0157:H8 strains were isolated from faecal specimens of 17 patients with diarrhoea

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*Escherichia coli* serogroup 0157 encompasses strains belonging to many different flagellar H types and with differing pathogenic properties. Some *E coli* 0157 strains cause porcine colibacillosis or extra-intestinal infections in man.<sup>1,2</sup> In recent years the most important development has been the recognition that strains of serotype 0157 are an important cause of

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in the United Kingdom; all had been isolated since 1984, with 11 strains isolated in 1990 or 1991. Twelve patients were aged between 1 and 15 months, one was aged 3 years, one was aged 16 years and the age of three patients was not given. Although other clinical details were rarely provided, the diarrhoea was stated to be bloody in one instance and accompanied by vomiting in two. Diarrhoea in the child aged 3 years had persisted for one month when the faecal specimen was taken. The patient aged 16 years had abnormal renal function; the diarrhoea was stated as being non-bloody.

Serotyping was performed with antisera against 173 O antigens and 56 H antigens using standard procedures.<sup>13</sup> Strains were tested for the ability to ferment sorbitol or rhamnose in Andrade peptone waters (containing 0.5% of the substrate). Colonial appearance was examined on SMAC agar and on SMAC agar containing rhamnose (0.5%) and cefixime (0.05 mg/l) (CR-SMAC), as described by Chapman *et al.*<sup>14</sup> Production of glucuronidase was assessed by the ability to hydrolyse 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG) using a previously described method.<sup>15</sup> Production of enterohaemolysin was tested on agar containing washed sheep erythrocytes.<sup>16</sup> All the strains were phage-typed using the extended scheme for VT positive *E coli* 0157 strains.<sup>17</sup>

Strains were tested for the ability to produce Vero cytotoxin using the Vero cell test and for adhesion to HEp-2 cells, using a six hour test in which the cells were washed after the first three hour period.<sup>18</sup> For the FAS test cells were fixed with formalin, permeabilised with Triton X-100, and treated with fluorescein isothiocyanate phalloidin.<sup>11</sup> The pattern of attachment was assessed using phase contrast microscopy<sup>19,20</sup> and attached bacteria were related to the appearance of fluorescing condensed actin using ultraviolet light.<sup>11</sup>

Strains were tested by colony hybridisation<sup>21</sup> for the presence of genes that may be associated with virulence. The probe for VT1 sequences was a 0.75 kilobase *HincII* fragment derived from a phage in strain H19 (serotype 026:H11)<sup>22</sup> and that for VT2 was a 0.85 kilobase *SmaI-PstI* fragment derived from the VT2 encoding phage of strain E32511 (serotype 0157:H non-motile).<sup>23</sup> The possession of sequences associated with AE lesions was detected with a 1 kilobase *KpnI-SalI* fragment, the *eae* probe.<sup>12</sup> To test for the presence of EPEC adherence factor (EAF) the EAF probe, a 1 kilobase *SalI-BamHI* fragment, was used.<sup>10</sup> All strains were also tested with the CVD419 probe, a 3.4 kilobase *HindIII* fragment of the 60 megadalton plasmid present in *E coli* 0157:H7 strains.<sup>4</sup> Hybridisation and washing for radioactive probes were as described previously.<sup>22</sup> The CVD419 probe was labelled with digoxigenin and used under the conditions described by Thomas *et al.*<sup>24</sup>

Strains were examined for plasmid DNA by gel electrophoresis of plasmid DNA prepared by the method of Birnboim and Doly.<sup>25</sup> Molecular sizes were measured relative to standard plasmids run on the same gel.

## Results

The results of tests with the 0157:H8 strains are shown in the table. Results deduced from published findings for similar tests with 0157:H7 strains are given for comparison.

All the 0157:H8 strains fermented sorbitol and rhamnose by the tube method within 24 hours. However, on SMAC medium, after 24 hours the appearance of the colonies varied: two strains gave colourless colonies, two gave pale pink colonies, while the remaining 13 strains gave dark red colonies. On CR-SMAC all colonies were dark red. The strains did not produce enterohaemolysin. All showed  $\beta$ -glucuronidase activity.

The 17 *E coli* 0157:H8 strains did not produce Vero cytotoxin in a tissue culture test or hybridise with probes specific for VT1 or VT2. All strains produced localised attachment to HEp-2 cells. The clusters contained more than 50 bacteria and the percentage of cells with such clusters ranged from 1–96, with a mean of 20. In the FAS test clusters were associated with the intense fluorescence indicative of a positive test. The strains hybridised with the *eae* probe but not with the EAF probe.

All 17 strains gave the same pattern of lytic reactions with the phages used in the typing scheme for VT positive *E coli* 0157 strains. The pattern did not match that of any of the 62 published phage-types of 0157 strains.

All the 0157:H8 strains carried a plasmid in the size range 72–78 megadaltons; additional plasmids from 38–50 megadaltons were present in four strains and a further three strains had small plasmids less than 2 megadaltons in size. The strains did not hybridise with the CVD419 probe.

## Discussion

The 0157:H8 strains had been referred to the DEP for VT testing because they had agglutinated with 0157 antiserum even though 13 of them should have produced red colonies on SMAC agar. Confluent or semi-confluent growth of fermenting strains on SMAC agar may often appear pale pink or colourless and

Properties of 17 strains of *E coli* 0157:H8 isolated from humans with diarrhoea and a comparison with properties of 0157:H7 strains as deduced from published reports\*

	Properties of strains of serotype	
	0157:H8	0157:H7
Fermentation of sorbitol (1 day)		
Peptone water	+	–
SMAC agar	+(15); –(2)	–
CR-SMAC agar	+	–
Production of		
VT	–	+
Enterohaemolysin	–	+
$\beta$ -glucuronidase	+	–
Adhesion to HEp-2 cells	LA	LA+
Reaction in FAS test	+	+
Hybridisation with probes for		
VT1 and/or VT2	–	+
EAF	–	–
<i>eae</i>	+	+
CVD419	–	+

\*Data from references<sup>4, 6, 11, 12, 14, 16, 23, 33</sup>

†Data from references<sup>11</sup> and <sup>26</sup>; other adhesion results were reported in reference<sup>27</sup>.

this may cause confusion. Two strains did produce colourless colonies on SMAC agar and so may well have been mistaken for VT positive 0157 strains. Only the VT positive 0157 strains would have appeared colourless after 24 hours on the recently described CR-SMAC medium.

The 0157:H8 strains produced localised adhesion to HEP-2 cells, were positive in the FAS test, and hybridised with the *eae* probe. Strains with these properties are considered to belong to the attaching and effacing class of *E coli* (AEEC)<sup>28</sup> which includes both EPEC and EHEC such as VT producing strains of 0157:H7.<sup>11 12 29</sup> These attaching and effacing but VT negative *E coli* 0157:H8 strains may represent another group of 0157 strains that are a putative cause of diarrhoea. Further studies are needed to assess the prevalence of such strains.

We have reported other strains of *E coli* that have the properties of AEEC but, like the 0157:H8 strains, do not hybridise with the EAF probe. These include VT negative strains belonging to traditional EPEC serogroups such as 0128,<sup>30 31</sup> and VT positive strains belonging to serogroups 05, 026, and 0103.<sup>30 32</sup> For several of these EAF negative strains, for example, E25253 (0128:H2) and S22-1 (0103:H2), loss of a plasmid resulted in a decrease in attachment to HEP-2 cells, although adhering bacteria still caused actin accumulation.<sup>30 31</sup> These results indicate that, as for EAF positive strains such as E2348/69 (0127:H6),<sup>10</sup> plasmid-encoded genes may have a role in the attachment of EAF negative strains. Similar results have been reported in some studies with EAF negative, VT positive 0157:H7 strains which carry the common 60 megadalton plasmid.<sup>27</sup> We do not know yet whether the 70 megadalton plasmid common to the EAF negative, VT negative 0157:H8 strains is similarly involved in attachment.

The 0157:H8 strains differed from VT positive 0157 strains in several respects. Enterohemolysin was produced by all 32 VT positive 0157 strains tested in the study of Beutin *et al.*<sup>16</sup> but the eight 0157:H8 strains did not produce enterohemolysin. Unlike most *E coli* strains, VT positive 0157 strains do not produce  $\beta$ -glucuronidase. The 0157:H8 strains resembled other VT negative 0157 strains<sup>15 33</sup> in possessing  $\beta$ -glucuronidase activity. However, the VT positive non-motile 0157 strains examined by Karch *et al.*,<sup>9</sup> that were atypically sorbitol fermenting, were also  $\beta$ -glucuronidase positive. The 0157:H8 strains did not hybridise with the CVD419 probe. From this result it could be concluded that the large 70 megadalton plasmid carried by these strains did not have a gene sequence homologous to CVD419 that was originally derived from the large 60 megadalton plasmid carried by an 0157:H7 strain.

The availability of 0157 antiserum for the investigation of cases of diarrhoea for the presence of VT positive strains of 0157 has occasionally resulted in the isolation of VT negative 0157:H8 strains. The presence of these strains in the faeces of patients with

diarrhoea reinforces the advice that 0157 strains should be sent to a reference laboratory for confirmatory tests such as VT production.

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