Characterisation of a Novel EAST-Negative Enteropathogenic *E. coli* strain Implicated in a Food-borne Outbreak of Diarrhoea in Adults

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Running title: Food-borne Outbreak of Enteropathogenic *E. coli*

Summary

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Enteropathogenic *Escherichia coli* (EPEC) is usually associated with outbreaks and sporadic cases of severe infantile diarrhoea in the developing world, and less commonly with sporadic cases in developed countries. Very little evidence indicates that EPEC is a food-borne pathogen for adults. In a previous study, two groups of adult travellers became ill, and *eae*$^{+}$ *E. coli* of serogroup O111 was isolated from affected individuals and epidemiologically linked to food consumption. Here the strain responsible was further investigated and characterised as an unusual atypical EPEC. PCR analysis of the designated type isolate showed the presence of the *rorf1* and *espB* genes of the LEE pathogenicity island, which was inserted at the chromosomal *selC* locus. The isolate was negative for the enteroaggregative *E. coli* EAST-1 toxin present in other strains of EPEC associated with food poisoning outbreaks. The strain adhered sparsely to HEp-2 cell monolayers in a diffuse manner, but fluorescent actin staining demonstrated that it was capable of inducing polymerisation of actin at the sites of bacterial attachment. Strain P2583 is the first EAST-negative EPEC to be confirmed as a cause of outbreaks of infection in adults following the consumption of contaminated food or water.

Key words: Enteropathogenic E. coli; food-borne illness; travellers diarrhoea; HEp-2 adherence assay; fluorescent actin staining

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Introduction

Enteropathogenic *Escherichia coli* (EPEC) was first identified as a causative agent of infantile diarrhoea in maternity and post-natal hospital settings in Western countries: while the number of such cases has declined dramatically, EPEC is still a major cause of infant morbidity and mortality in the developing world (1). Unlike other pathotypes of diarrhoeagenic *E. coli*, EPEC rarely causes serious disease in older children or adults, but outbreaks continue to occur in children under five in many countries especially in nursery and hospital settings (2-4).

Common to attaching-effacing pathogens such as EPEC and Enterohemorrhagic *E. coli* (EHEC) is the chromosomal locus of enterocyte effacement (LEE) (5), which encodes all genes necessary to cause the characteristic structural and biochemical changes seen during infection (6). Additionally, the presence or absence of the plasmid-encoded bundle-forming pili subdivides EPEC into respectively typical and atypical strains: the latter are much more heterogeneous, and considered to be more closely related to EHEC than to typical EPEC (7, 8).

Sporadic cases of EPEC infection have been described in older children and adults, but these are relatively rare (9-12), additionally adults may be asymptomatic carriers of EPEC (13, 14). Outbreaks amongst adults of EPEC infection attributed to food or water consumption are extremely rare. In two such instances the strains believed to be responsible were shown to be atypical EPEC which also carried the *astA* gene for the Enteroaggregative *E. coli* EAST-1 toxin, thus the symptoms may have resulted from this toxin alone, rather than the characteristic attaching and effacing properties of EPEC (15, 16). A further outbreak putatively attributed to EPEC prompted the current investigation. Those affected were members of two groups of ‘weekend tourists’ to Northern France from the Sheffield area of the UK, many of whom reported gastrointestinal illness on their return. Epidemiological

investigation implicated food from a restaurant at which both groups had eaten (17). *E. coli* of serotype O111:H9 was considered the potential pathogen, and was classified as EPEC solely on the basis of hybridisation to an *eae* gene probe and absence of genes for shiga toxins. The *eae* gene is not absolutely unique to EPEC (18, 19), and the subsequently identified intimin type (intimin ξ) of the putative EPEC (20) is commonly associated with shiga toxin-producing *E. coli* (21). The very unusual involvement of an EPEC strain in a presumptive food poisoning outbreak, coupled with its EHEC-like intimin prompted us to revisit the original identification and to investigate whether it displayed characteristic EPEC properties, or was another pathotype entirely, or simply a vehicle for the EAST toxin.

Materials and Methods

Bacteria. Ten isolates of *E. coli*, P2582-86 and P2594-98 from the first and second affected groups of travellers respectively, were provided by Dr Peter Chapman, Health Protection Agency, Sheffield, U.K. Isolates were grown on Nutrient Agar and stored in glycerol broth at -70°C. Control strains for adherence and PCR assays were locally adherent EPEC strain E2348, DAEC C872, EAEC 17-2 and 042 and the non-pathogenic *E. coli* DH1.

Plasmid Isolation. Plasmids were extracted from the isolates using a rapid alkaline lysis and analyzed by agarose gel (0.8% w/v) electrophoresis.

Polymerase Chain Reaction and Cloning. Sequences of primers to detect the presence of the *rorf1* and *espB* genes, to detect the insertion point of the LEE pathogenicity island in the chromosome, and to investigate the structure of the left-hand end of the LEE are listed in Table 1. LEE specific primers were based on the sequence of the E2348 LEE genes; those for the amplification of *selC* and *pheU* were those of Sperandio *et al* (22). PCR amplifications were performed in a Peltier thermalcycler (MJ Research) with Promega 'Mastermix' in 20µl reaction volume containing 0.01nmol of each primer. Template DNA was obtained by boiling 100µl of sterile distilled water inoculated with half of one colony of *E. coli*; 0.5µl of the suspension was used per reaction. Amplification protocols consisted of denaturation at 92˚C for 2 minutes, 30 cycles of denaturation at 92˚C for 1 minute, annealing temp (see table 1) for 1 minute and extension at 72˚C for 1 minute, followed finally by extension at 72˚C for 5 minutes. PCR products were detected by agarose gel electrophoresis in TBE buffer. Amplicons from the left end of the LEE were purified using a Qiaquick PCR purification kit

(Qiagen) and cloned into plasmid pGEM-T (Promega) for nucleotide sequencing (GATC-Biotech, Germany).

Adherence Assays and FAS test. HEp-2 cell monolayers were grown to 80-90% confluence in RPMI with HEPES and glutamine (Gibco) supplemented with 10% (v/v) foetal calf serum (Biosera) and penicillin and streptomycin on 16mm glass cover slips in 12 well tissue culture plates at 37°C. Adherence assays were carried out as described by Cravioto *et al* (23). Briefly, monolayers were washed three times with RPMI, 2ml of fresh RPMI containing 0.5% (w/v) D-mannose was added to each well, then inoculated with 50µl of overnight bacterial culture grown in nutrient broth. After three hours the monolayers were washed, fixed with methanol and stained with Giemsa (BDH) for 30 minutes. After further washing, the cover slips were washed with Histoclear (National Diagnostics) and acetone. Cover slips were mounted in Histomount and adherence patterns observed by light microscopy.

For the fluorescent actin staining (FAS) test (24), monolayers were prepared and infected as above, than after 3 hours were fixed for 20 minutes with 3% formalin. Monolayers were washed with PBS and treated with 0.1% Triton X-100 in PBS for 5 minutes to permeabilise the cells. After three washes with PBS, the cover slips were treated with 5µg/ml tetramethyl-rhodamine isothiocyanate (TRITC)–phalloidin (Sigma) in PBS for 20 minutes to stain specifically filamentous actin. The cover slips were then washed in PBS and mounted in 50:50 glycerol-PBS. The specimens were examined by incident light fluorescence microscopy using a Nikon Eclipse 80i fluorescent microscope.

Results

Homogeneity of isolates from the two outbreaks.

Plasmid preparations of all 10 isolates revealed identical plasmid profiles (data not shown). Similarly, antibiotic resistance assays by disc diffusion also showed that the strains appeared to be identical, being resistant to ampicillin and carbenicillin, but sensitive to tetracycline, colistin sulphate, sulphafurazole and gentamicin. This confirms that only one strain was responsible for the outbreaks of disease among both sets of travellers, and thus a single source, believed to be food eaten at a restaurant visited by both parties (17). We have designated P2583 as the type isolate for this strain.

PCR analysis of the outbreak strain.

Previous work had identified the *E. coli* responsible for the restaurant outbreaks as an EPEC on the basis of hybridization with a non-type specific probe for *eae*. Genomic DNA extracted from isolates P2583 and P2597 was used as templates for PCR to investigate the extent of the EPEC associated genes. Confirmation of the presence of the EPEC and EHEC associated LEE pathogenicity island was obtained by specific amplification of genes located at or near the extremities of the LEE, namely *rorf1* and *espB*, located respectively at the 'left' and 'right' ends (25). Both targets produced amplicons of the predicted size (Figures 1a and 1b), and these results coupled with the previous detection of the *eae* gene strongly suggest the presence of the entire LEE. The insertion point in the *E. coli* chromosome was established with primers directed at the *selC* and *pheU* tRNA genes. No amplification was obtained with primers targeting *selC* with P2383, P2597 or the control EPEC strain E2348, which is known to have the LEE inserted at this point. Conversely, amplification products were obtained with *pheU* specific primers with P2383, P2597 and E2348 DNA, but not with the control K798, which has the LEE inserted in *pheU* (unpublished data) (Figure 2). In two previous studies on

outbreaks of EPEC-mediated diarrhoeal illness in adults the strains responsible possessed the gene (*astA*) encoding the enteroaggregative *E. coli* heat stable toxin. PCR directed against this gene also amplified the predicted product two positive control EAEC strains, 17-2 and 042, but failed to amplify DNA from two of the outbreak isolates (Figure 1c). (26) have demonstrated that for some strains of atypical EPEC the 'left' end of the LEE shows considerable structural variability, with additional insertion sequences and/or deletions. In the case of P2583, overlapping PCR analysis over 4.5kb from the junction with *selC* to the *ler* gene demonstrated that the structure of the LEE is identical to that of the prototype typical EPEC strain E2348, with only a very small number of base differences (data not shown).

Adherence to HEp-2 cells and FAS test.

Three hour adherence assays using isolate P2583 were performed simultaneously with control assays for localised adherence (E2348), diffuse adherence (C872) and aggregative adherence (042) in order for a direct comparison of adherence patterns to be made. Under these conditions, the control strains produced the anticipated patterns. However, the outbreak isolated adhered sparsely to the HEp-2 cells, with relatively few discrete bacteria associated with each cell (Figure 3) and no evidence of the microcolonies which characterise typical *bfp*$^{+}$ EPEC. Some bacteria also appeared to be adhering to the coverslip support. The low levels of adherence made it difficult to unequivocally assign any particular pattern of adherence, but as there was no obvious association between individual bacteria it was considered that the type of adherence was best described as 'sparse diffuse'. No aggregative adherence was seen, either to the HEp-2 cells, or to the glass coverslip in the honeycomb formations seen with many strains of EAEC (27).

The FAS test has previously demonstrated its usefulness in detecting polymerised actin beneath adherent microcolonies of locally adherent EPEC (24) where the amount of actin polymerization is amplified by the relative high density of the bacteria in the microcolony. With the sparse adherence seen with the restaurant isolate, it was anticipated that the fluorescence in the assay would be limited to that induced by individual bacteria. Using a slight excess of FITC-phalloidin, small bright spots of fluorescence indicating actin accumulation were detected, the distribution of which appeared to correspond to that of the adherent bacteria (Figure 4), implying that the strain is able to induce actin rearrangements.

Discussion

Food or water associated outbreaks of EPEC infection are noteworthy by their rarity: the organism is not usually considered as a plausible aetiologic agent in the diagnosis of such cases, nor is it linked to travellers diarrhoea. However, a previous study had reported that two outbreaks of gastrointestinal illness among adult weekend tourists from the UK to Northern France were epidemiologically linked to the consumption of food at one restaurant, and implicated the causative agent as EPEC on the basis of serogroup (O111), hybridisation with a non-type specific *eae* probe, and absence of genes for Shiga-like toxins (17). Only two other outbreaks of EPEC-mediated gastrointestinal illness, in Japan (16) and the USA (15), linked to the consumption of contaminated food or water have been described, in both cases these were atypical EPEC also carrying the *astA* gene encoding the EAST-1 toxin, which is commonly found in enteroaggregative *E. coli*. In contrast to EPEC, EAEC has been implicated as a major cause of gastrointestinal illness in travellers (28, 29), and has also been found in foods such as meat and vegetables (30). The unusual circumstances of these outbreaks, and the improbability of the cause being EPEC prompted us to revisit the original identification and to investigate whether the strain(s) displayed characteristic EPEC properties or was of another pathotype carrying the *eae* gene (31).

All 10 isolates available showed the same antibiotic resistance and plasmid profiles, confirming that only one strain was responsible for both outbreaks of disease, and that this was present throughout the two weeks separating the visit of the two parties to the restaurant.

The identity of the strains as an attaching-effacing pathogen was confirmed by demonstrating that two genes near or at the extremities of the LEE were present. The genetic organization of the majority of the LEE is very highly conserved in EPEC and EHEC, with all the currently identified genes and open reading frames invariably being present, though some variation in the order of some of the genes is seen in other attaching effacing pathogens,

including *Citrobacter rodentium* (32). Of the two genes used here as LEE markers, the *espB* gene lies close to the 'right hand' end of the LEE (25) and encodes an effector protein translocated through, but also possibly being part of, the LEE encoded Type Three Secretion System translocon (33, 34). *rorf1* lies at the 'left hand' end of the LEE in EPEC and EHEC. Its function is as yet unknown, but its presence as part of the LEE of all attaching-effacing pathogens implies a role in pathogenesis. Successful amplification of both target genes implies that the LEE is present in its entirety. Only three LEE insert points in the *E. coli* genome have been documented, in the case of P2583 the LEE lies within the *selC* locus. Taken with the other results, this data indicates that the strain is an atypical EPEC. These strains are rather more heterologous than typical EPEC, and Muller *et a.l* (2010) have demonstrated that in some strains at least, there is variability at the left hand end of the LEE, with IS insertions and gene rearrangements. For P2383, overlapping PCR analysis and sequencing of the resultant amplicons demonstrated that the P2397 LEE is virtually identical to that of the prototype typical EPEC, E2348.

The failure to amplify the *astA* gene encoding the EAST-1 toxin is significant. The EPEC strains responsible for two previously described food-associated outbreaks in adults (15, 16) both carried the *astA* gene. EAST-1 is associated with Enteroaggregative *E. coli* (EAEC) though it is not ubiquitous among strains of this pathotype, and can be found in other diarrhoeagenic *E. coli* (35). Indeed, in a study of Brazilian infants, Dulguer *et al.* (36) showed a strong association with the presence of *astA* gene in atypical EPEC and diarrhoeal illness. In contrast, Vila *et al.* (37) found that in a survey of Spanish travellers to developing countries who suffered diarrhoea that the presence of EAST was not in itself a marker of disease causing strains, unless an additional virulence factor was present. It is possible that in the two other food-borne outbreaks noted above, EAST may have contributed to the severity

of the symptoms, but for P2583, the absence of EAST implies that the illness was caused by the ability of the strain to induce attaching-effacing lesions in the gut.

From the presence of the LEE, the strain is genotypically EPEC, however the possibility that the strain was an EAST-negative enteroaggregative *E. coli* that also carried this pathogencity island was also investigated. EAEC are a heterologous collection of strains, currently defined phenotypically by their aggregative adherence to HEp-2 and related cell lines (27). In the adhesion assays, P2583 was very sparsely adherent, with no evidence of the microcolonies which characterise typical *bfp*$^+$ EPEC. The number of adherent bacteria per cell was much less than for a known DAEC, with the individual bacteria being scattered widely across the surface of the HEp-2 cells. No aggregative adherence was seen, either to the HEp-2 cells, or to the glass coverslip in the honeycomb formations seen with many strains of EAEC.

The relative poor adherence of the restaurant strain meant that the results of the FAS test were not as clear as is seen with locally adherent EPEC. With the latter, the intense spots of fluorescence correspond to polymerised actin produced by numbers of bacteria contacting the plasma membrane in a small area. With P2583 spots of fluorescence are caused by individual bacteria, hence are much smaller and less easy to detect. It was apparent that there were spots of fluorescence on the cells after actin staining, the distribution of which was comparable with that of the bacteria over the surface of the HEp-2 cell, implying that the strain is able to induce actin rearrangements.

This study has shown unambiguously that the strain associated with the two outbreaks of gastrointestinal illness in travellers to Northern France is an atypical EPEC. Initially dismissed as of low virulence, atypical EPEC are more commonly isolated then typical EPEC in some areas of the world (18, 38), and remain a serious threat to health in infants. They have been associated with sporadic adult illness, but reports of adult outbreaks are rare. EPEC

of serotype O111:H9 type has been reported once before as a cause of an outbreak in older children and adults, but the strain involved was not fully characterised, nor was the source of the infection identified (39). Without further analysis, for instance by MLST it is impossible to say whether that strain (921-B4) and P2583 are closely related. This current study represents the first of a true EPEC strain which does not potentially produce the EAST-1 toxin causing illness in adults linked to the consumption of food. The rarity of EPEC as a cause of adult outbreaks may lead to it being overlooked by diagnostic laboratories investigating reports of food-associated illness, so it is possible that this serotype may have been responsible for other outbreaks. Intimin ξ EPEC is usually associated with cattle, but the exact food source of the strain isolated in France was never established, and unfortunately the restaurant had already closed by the time of the investigation into the second outbreak (17).

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Table 1. Primers used for amplification of genes in PCR assays.

Target Gene	Forward Primer Reverse Primer	Annealing Temp (°C)	Amplicon size (bp)
espB			
EspB1	GTCAGCGGTCGAGTTTA	51	990
EspB2	ATTAAACGTATCGACCATG		
rorf1			
HNO3	AACAATTACCAAATACGGTG	50	672
HNO4	GCGAGGATATCAGGTTCAC		
astA			
ASTA1	GCCATCAACACAGTATATCC	52	106
ASTA2	GAGTGACGGCTTTGTAGTCC		
selC			
SelCf	GAGCGAATATTCCGATATCTGGTT	52	527
SelCr	CCTGCAAATAAACACGGCGCAT		
pheU			
PheUf	CATCGGCTGGCGGAAGATAT	52	308
PheUr	CGCTTAAATCGTGGCGTCC		
selC – rorf1			
LLE1F	GCGTGAGGTATTTGCATATA	56	919
LLE1R	TTGAGATGCTTGACCTGAC		
rorf1 – espG			
HNO5	TGAGTGTTCTTGAGGTGATTTTT	53	769
EspG1	GCAAATGCACAAATGTTGCT		
espG			
EspG2	TGTCTTTAAGTCCTAGTGCATCG	53	857
EspG3	CTGACGCATCACTTGCAATC		
espG – ERIC			
LLE3F	GCAAGTGATGCGTCAGCCATA	58	720
ERIC2F	AAGTAAGTGACTGGGGTGAGCG		
ERIC – orf2			
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	57	870
orf2R	TTCGTCTTCCAGCTCAGTTATCG		

Figure 1

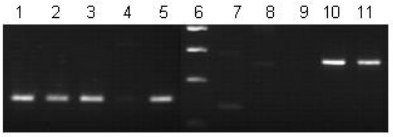

Figure 2

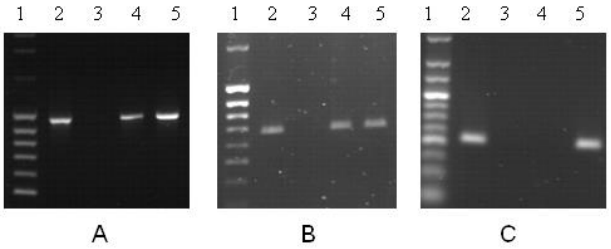

Figure 3

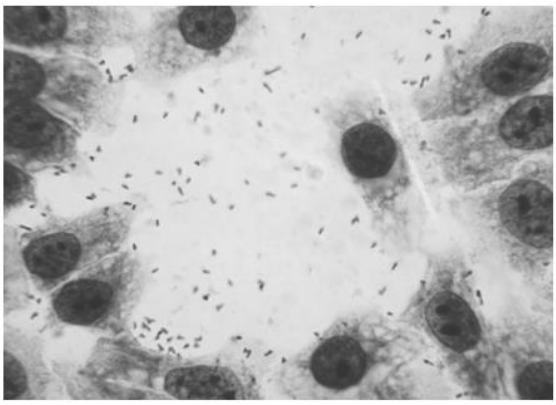

Figure 4

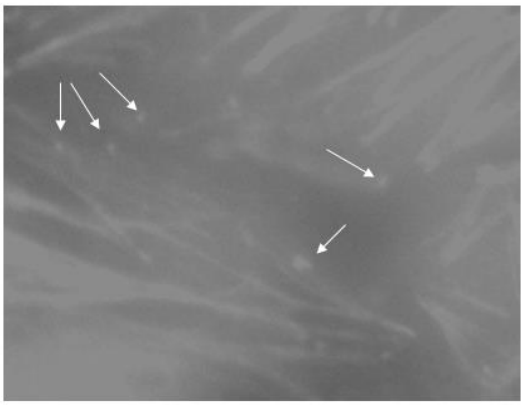

Figure legends

Figure 1. Detection of the point of insertion of the locus of enterocyte effacement in the chromosome of the outbreak isolate and control strains. Where there is no insert, PCR products of 308bp and 527bp should result for amplification with *pheU* and *selC* specific primers. Lanes 1-5 with *pheU* specific primers, lanes 7-11 with *selC* specific primers. Lanes 1 and 7 EPEC E2348; lanes 2 and 8 P2583; lanes 3 and 9 P2597; lanes 4 and 10 EPEC K798; lanes 5 and 11 DAEC C872; lane 6 molecular weight marker (Hyperladder I, Bioline).

Figure 2. Detection of the genes at the extremities of the locus of enterocyte effacement and presence of the *astA* gene encoding the EAST-1 enterotoxin in the DNA of the outbreak isolate and control strains

(A) Amplification with *espB* specific primers. Lane 1 molecular size marker (Gene O'Ruler, Fermentas); lane 2 EPEC E2348; lane 3 *E. coli* DH1; lane 4 P2583; lane 4 P2597.

(B) Amplification with *rorf1* specific primers. Lane 1 molecular size marker (Gene O'Ruler, Fermentas); lane 2 EPEC E2348; lane 3 *E. coli* DH1; lane 4 P2583; lane 4 P2597.

(C) Amplification with *astA* specific primers. Lane 1 molecular size marker (Hyperladder V, Bioline); lane 2 EAEC 17-2; lane 3 P2583; lane 4 P2597; lane 5 EAEC 042.

Figure 3. Adherence of isolate P2583 to HEp-2 cells. Bacteria were incubated with HEp-2 cell monolayers for 3 hours in the presence of D-mannose, washed and stained with Giemsa. Bacteria adhere sparsely to the cells and to the glass of the coverslip, no obvious patterns of adherence or association of bacteria are evident.

Figure 4. Fluorescent actin staining of HEp-2 cells infected for 3 hours with isolate P2583. Bacteria were incubated with HEp-2 cell monolayers for 3 hours, then permeabilised and stained 5μg/ml solution TRITC-phalloidin in PBS for 20 minutes. Small spots of fluorescence not associated with cytoskeletal filaments represent the location of areas of polymerised actin beneath individual adherent bacteria.