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# Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study

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## Summary

**Background:** In an ongoing outbreak of haemolytic uraemic syndrome and bloody diarrhoea caused by a virulent *Escherichia coli* strain O104:H4 in Germany (with some cases elsewhere in Europe and North America), 810 cases of the syndrome and 39 deaths have occurred since the beginning of May, 2011. We analysed virulence profiles and relevant phenotypes of outbreak isolates recovered in our laboratory.

**Methods:** We analysed stool samples from 80 patients that had been submitted to the National Consulting Laboratory for Haemolytic Uraemic Syndrome in Münster, Germany, between May 23 and June 2, 2011. Isolates were screened with standard PCR for virulence genes of Shiga-toxin-producing *E coli* and a newly developed multiplex PCR for characteristic features of the outbreak strain (*rfb*<sub>O104</sub>, *fliC*<sub>H4</sub>, *stx*<sub>2</sub>, and *terD*). Virulence profiles of the isolates were determined with PCR targeting typical virulence genes of Shiga-toxin-producing *E coli* and of other intestinal pathogenic *E coli*. We sequenced *stx* with Sanger sequencing and measured Shiga-toxin production, adherence to epithelial cells, and determined phylogeny and antimicrobial susceptibility.

**Findings:** All isolates were of the HUSEC041 clone (sequence type 678). All shared virulence profiles combining typical Shiga-toxin-producing *E coli* (*stx*<sub>2</sub>, *iha*, *lpf*<sub>O26</sub>, *lpf*<sub>O113</sub>) and enteroaggregative *E coli* (*aggA*, *aggR*, *set1*, *pic*, *aap*) loci and expressed phenotypes that define Shiga-toxin-producing *E coli* and enteroaggregative *E coli*, including production of Shiga toxin 2 and aggregative adherence to epithelial cells. Isolates additionally displayed an extended-spectrum  $\beta$ -lactamase phenotype absent in HUSEC041.

**Interpretation:** Augmented adherence of the strain to intestinal epithelium might facilitate systemic absorption of Shiga toxin and could explain the high progression to haemolytic uraemic syndrome. This outbreak demonstrates that blended virulence profiles in enteric pathogens, introduced into susceptible populations, can have extreme consequences for infected people.

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## Introduction

Starting in early May, 2011, an outbreak of haemolytic uraemic syndrome and bloody diarrhoea caused by *Escherichia coli* O104:H4 has spread throughout Germany.<sup>1</sup> As of June 20, 2011, 810 cases of haemolytic uraemic syndrome, 2684 non-haemolytic uraemic syndrome cases, and 39 deaths have been reported by the Robert Koch Institute and European Centre for Disease Prevention and Control. The particular features of this outbreak are the predominant involvement of adult women<sup>1</sup> and common severe neurological complications, such as encephalopathy and epileptic seizures. Almost 100 infections with 39 cases of haemolytic uraemic syndrome caused by the outbreak strain have been reported in other European states including Austria, the Czech Republic, Denmark, France, Greece, Luxemburg, the Netherlands, Norway, Poland, Spain, Sweden (including one death), and the UK and in Canada and the USA—most non-German patients seem to be travellers returning from Germany. In the past 15 years, only a single *E coli* O104:H4 has been isolated from a patient with haemolytic uraemic syndrome in Germany<sup>2</sup> (HUSEC041, which is included in the collection of the National Consulting Laboratory for Haemolytic Uraemic Syndrome, University of Münster, Münster, Germany). A single additional case of the syndrome associated with *E coli* O104:H4 has also been reported from a woman in Korea.<sup>3</sup> To gain insight into the apparently augmented virulence of the outbreak strain, we analysed virulence profiles and relevant phenotypes of outbreak isolates recovered in our laboratory. We also determined phylogenetic relations of the isolates and their antimicrobial susceptibilities.

## Methods

### Stool samples and analysis

The first stool sample from the suspected outbreak, originating from a patient with haemolytic uraemic syndrome, arrived at the National Consulting Laboratory for Haemolytic Uraemic Syndrome in Münster, Germany on May 23, 2011. By June 2, stools from 79 additional patients including 66 with haemolytic uraemic syndrome and 13 with bloody diarrhoea hospitalised in 22 hospitals in 17 different German cities distributed in nine of 16 federal states were received for microbiological analysis. The strains were isolated by use of stool enrichment in GN broth Hajna, plating the enriched cultures on extended-spectrum  $\beta$ -lactamase (ESBL) agar plates (chromID ESBL, bioMérieux, Nürtingen, Germany) and cefixime-tellurite sorbitol MacConkey agar (CT-SMAC, Oxoid, Wesel, Germany), and subsequent screening for *stx*, *eae*, and EHEC-*hlyA* with our standard PCR protocol.<sup>4</sup> In addition, we developed and used multiplex PCR targeting typical molecular features of the outbreak strain (*rfb*<sub>O104</sub>, *fliC*<sub>H4</sub>, *stx*<sub>2</sub>, and *terD*; figure 1 and table 1). Multiplex PCR was done with the REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich, Taufkirchen, Germany) in a total volume of 25  $\mu$ L containing 2  $\mu$ L of bacterial DNA. PCR conditions included initial denaturation at 94°C for 5 min, 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 60 s), and extension (72°C, 60 s), and final extension at 72°C for 5 min. *fliC* genes of the isolates encoding the H antigen were subtyped with *fliC* restriction fragment length polymorphism (RFLP).<sup>7</sup> HUSEC041 (O104:H4) and HUSEC037 (O104:H21) strains were used for comparison with the outbreak isolates.

### Virulence analysis and antimicrobial susceptibility

Virulence profiles of the outbreak isolates were compared with those of HUSEC041 by use of a set of PCR analyses targeting a broad range of virulence and fitness genes of Shiga-toxin producing *E coli* [5], [6], [7], [8], [9], [10], [11], [12] and [13] that typically cause haemolytic uraemic syndrome,<sup>14</sup> such as those encoding toxins (*stx*<sub>1</sub>, *stx*<sub>2</sub>, EHEC-*hlyA*, *cdt*, *subAB*), serine proteases (*espP*), adhesins (*eae*, *iha*, *lpfA*<sub>O26</sub>, *lpfA*<sub>O113</sub>, *lpfA*<sub>O157-01141</sub>, *lpfA*<sub>O157-01154</sub>, *saa*, *sfpA*), iron acquisition system encoded on high pathogenicity island (marker genes *irp2* and *fyuA*), and tellurite resistance (*terD* as a marker for the *ter* cluster). All isolates were PCR-tested for virulence loci typical for other intestinal pathogenic *E coli* including enteropathogenic (*bfpA* encoding structural subunit of bundle-forming pili), enterotoxigenic (*elt* and *estI* encoding heat-labile and heat-stable enterotoxin, respectively), enteroinvasive (*ial*, a marker for the virulence plasmid plnv), and enteroaggregative *E coli* (*astA*, *sen*, *set1*, and *pic* encoding toxins, *aggA*, *aafA*, *agg3A*, and *agg4A* encoding adhesins, *aggR* encoding transcription regulator, *aap* encoding dispersin, and *aat* as a marker for enteroaggregative *E coli* virulence plasmid). [15], [16], [17], [18], [19] and [20]

*stx* genes of four outbreak isolates from four different cities were amplified and Sanger sequenced; resulting sequences were analysed with the Ridom TraceEditPro Software (Ridom GmbH, Münster, Germany) and homologies were sought in GenBank.

The isolates were confirmed as *E coli* (API 20 E, bioMérieux) and serotyped.<sup>21</sup>  $\beta$ -D-glucuronidase activity was assessed by use of nutrient agar with 4-methylumbelliferyl- $\beta$ -D-glucuronide (Becton Dickinson, Heidelberg, Germany). Production of enterohaemorrhagic *E coli* haemolysin was sought on enterohaemolysin agar (Sifin, Berlin, Germany), and production of  $\alpha$  haemolysin on Columbia blood agar (Heipha, Heidelberg, Germany). Resistance to tellurite was determined by ability to grow on CT-SMAC. Shiga-toxin production was identified with the Vero cell cytotoxicity assay.<sup>4</sup> Adherence to human intestinal epithelial cells (HCT-8; ATCC CCL-244) was tested as described previously<sup>22</sup> by use of semiconfluent monolayers incubated with the strains for 3 h in the presence of 0.5% D-mannose. Bacterial adherence patterns were examined with phase-contrast microscopy (AxiomagerA1; Zeiss, Jena, Germany) of washed, fixed, and Giemsa-stained cells.

Antimicrobial susceptibility was determined by use of the Vitek 2 system version 05.02 (bioMérieux). Minimum inhibitory concentrations were interpreted in accordance with EUCAST 2010 guidelines.<sup>23</sup>

## Genetic analysis

Multilocus sequence typing (MLST) was done by sequencing internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*).<sup>24</sup> The alleles and sequence type (ST) were assigned in accordance with the *E coli* MLST databases at the Environmental Research Institute, University College Cork, Ireland. In addition to MLST, all strains were subjected to partial *gnd* sequencing (627 bp internal *gnd* gene fragment, positions 397–1023 in strain W3110, GenBank accession number AP009048) in accordance to the protocol of Gilmour and colleagues<sup>25</sup> Three reference *E coli* O104 strains including HUSEC041 (O104:H4), HUSEC037 (O104:H21), and enteroaggregative *E coli* strain 55989 (O104:H4; GenBank accession number NC\_011748) were included as controls. The partial *gnd* sequence enables prediction of the O-phase of *E coli*.<sup>25</sup>

## Role of the funding source

The funding institutions had no role in the study design, data collection, analysis and interpretation, writing the Article or decision for publication. The corresponding author had full access to all the data in the study and final responsibility for the decision to submit for publication.

## Results

The first outbreak strain (LB226692) was isolated in the German National Consulting Laboratory for Haemolytic Uraemic Syndrome in Münster on May 24, 2011. Molecular analysis of the isolate with PCR detected an *stx*<sub>2</sub>-positive, *eae*-negative Shiga-toxin-producing *E coli*, which was further characterised by sequence analysis of *gnd*, *fliC* RFLP, and MLST as *gnd*<sub>O104</sub>, *fliC*<sub>H4</sub>, and ST678. Moreover, the strain grew on CT-SMAC and possessed *ter* cluster encoding tellurite resistance. These characteristics of the isolate enabled us to develop the multiplex PCR targeting the typical molecular features of the strain (*rfb*<sub>O104</sub>, *fliC*<sub>H4</sub>, *stx*<sub>2</sub>, and *terD*; figure 1), which we then used for identification of the subsequent outbreak isolates. We proved specificity of the multiplex PCR by showing that, among the 42 reference strains of the HUSEC collection and 100 additional Shiga-toxin-producing *E coli* of various serotypes as well as *E coli* from normal intestinal flora, only HUSEC041 gave amplicons for all the four loci targeted by the multiplex PCR.

All 80 outbreak isolates possessed *rfb*<sub>O104</sub> belonging to the O104 biosynthetic cluster and *fliC*<sub>H4</sub> belonging to the H4 antigen biosynthetic cluster, as determined by multiplex PCR (figure 1). *gnd* of all isolates was identical in the 627 bp sequenced fragment and it was identical to *gnd* of the two O104 reference strains in the HUSEC collection (HUSEC037 and HUSEC041) and of strain 55989 (GenBank accession number NC\_011748), corroborating the O104 lipopolysaccharide phenotype. Presence of *fliC*<sub>H4</sub> was verified using RFLP. All 80 isolates belonged to serotype O104:H4. All of them

produced  $\beta$ -D-glucuronidase, fermented sorbitol and were tellurite resistant, and none produced enterohaemorrhagic *E coli* haemolysin or  $\alpha$  haemolysin. All 80 isolates belonged to ST678, as does HUSEC041. This sequence type is unique to serotype O104:H4 among Shiga-toxin-producing *E coli*. On the basis of MLST, *gnd* sequence, and presence of *fliC<sub>H4</sub>* we concluded that the isolates were a single clone.

Each of the 80 outbreak isolates possessed virulence genes typical of Shiga-toxin-producing *E coli*, including *stx<sub>2</sub>* encoding the toxin, *iha*, *lpf<sub>O26</sub>*, and *lpf<sub>O113</sub>* encoding adhesins, and the high pathogenicity island originally identified in *Yersinia* and encoding an iron uptake system;<sup>13</sup> *eae* encoding adhesin intimin<sup>15</sup> was not present (table 2). The *stx<sub>2</sub>* sequence was identical in four randomly chosen outbreak isolates and was 99% homologous to *stx<sub>2</sub>* sequence of prototypic Shiga-toxin-producing *E coli* O157:H7 strain EDL933 (GenBank AE005174), with one synonymous nucleotide change in each subunit gene (*stxA<sub>2</sub>* and *stxB<sub>2</sub>*). Moreover, all isolates possessed typical molecular characteristics of enteroaggregative *E coli*,<sup>[15], [16], [17], [18], [19] and [20]</sup> including the virulence plasmid (pAA), *aggA* encoding structural subunit of aggregative adherence fimbriae I (AAF/I), *aggR* encoding the enteroaggregative *E coli* global regulator AggR, *aap* encoding dispersin, *set1* encoding *Shigella* enterotoxin 1, and *pic* encoding an autotransporter serine protease (protein involved in intestinal colonisation). None of the isolates contained *astA* encoding enteroaggregative *E coli* heat-stable enterotoxin 1 (table 2). The absence of *astA* and the presence of the genes encoding AAF/I fimbriae differentiate, among the 34 alleles investigated, the outbreak isolates from HUSEC041, which contains *astA* as well as *agg3A* encoding the structural subunit of AAF/III fimbriae (table 2). These loci are also present in the reference enteroaggregative *E coli* strain 55989 (table 2). The results of the PCR analyses are concordant with the draft genome data of an outbreak isolate LB226692 (NCBI accession number AFPS01000000) after in-silico analysis of primer binding sites and the resulting fragment length determination.

In addition to harbouring a mixture of virulence loci of two different intestinal *E coli* pathotypes, all O104:H4 outbreak isolates also express the phenotypes that define Shiga-toxin-producing *E coli* and enteroaggregative *E coli*,<sup>[15] and [16]</sup> specifically production of Shiga-toxin 2 (Vero cell cytotoxicity titres 64–1024; median 256) and the aggregative (so-called stacked-brick) adherence pattern on intestinal epithelial cells (figure 2). The aggregative adherence phenotype (figure 2) combined with Shiga-toxin 2 production (Vero cell titre 64) are also displayed by HUSEC041. Virulence loci typically found in other intestinal pathogenic *E coli* including enteropathogenic, enteroinvasive, and enterotoxigenic *E coli* are absent from the O104:H4 outbreak isolates and from HUSEC041 (table 2).

All isolates have an ESBL phenotype—ie, they are resistant to all penicillins and cephalosporins and susceptible to carbapenems (ertapenem, imipenem, meropenem). This resistance pattern is typical for CTX-M-1 group  $\beta$  lactamases and corroborates the already reported findings of the CTX-M-15 genotype.<sup>26</sup> Furthermore, all strains are resistant to co-trimoxazole (trimethoprim–sulfamethoxazole) and susceptible to fluoroquinolones (ciprofloxacin) and aminoglycosides (gentamicin, tobramycin). HUSEC041 did not have the ESBL phenotype.

## Discussion

The genotypes, phenotypes, and phylogeny of the 80 outbreak isolates demonstrate that the *E coli* O104:H4 outbreak strain is a clone that combines virulence potentials of two different pathogens: Shiga-toxin-producing *E coli* and enteroaggregative *E coli* (panel). HUSEC041, isolated in 2001 from a child with haemolytic uraemic syndrome in Germany, also had this virulence combination.

Panel. Research in context

### Search strategy

We searched the PubMed database for papers published in English between Jan 1, 1990, and June 14, 2011, with the search terms “O104”, “O104:H4”, “HUS” (haemolytic uraemic syndrome), “enteroaggregative”, or “enterohaemorrhagic”. No articles provided a comprehensive characterisation of the enterohaemorrhagic *Escherichia coli* O104:H4 causing a large outbreak in Germany.

### Interpretation

*E coli* O104:H4, which has characteristics and genetic loci found in Shiga-toxin-producing *E coli* and enteroaggregative *E coli*, is unprecedented among enteric pathogens in its virulence. This isolate belongs to the rare sequence type 678 and produces Shiga toxin 2 and aggregative adherence to intestinal epithelial cells. This pathogen, and this outbreak, will have profound implications for disease detection, reporting, food safety, and our understanding of microbial pathogenesis.

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Isolation of this rare *E coli* O104:H4 serotype in many samples and in a short time confirms that this is the outbreak strain; however, we do not have access to the entire strain collection from this outbreak because no single laboratory has statutory authority for the analysis or possession of these isolates. Because of the rapidly increasing number of infected people and the severity of the disease, identification and profiling of the outbreak strain as expeditiously as possible was essential. In this race against time, sequence analysis of *gnd*, identification of ST678 (which is unique to serotype O104:H4 among Shiga-toxin-producing *E coli*, until now reported only in the HUSEC041 reference strain from 2001), and identification of *fliC<sub>H4</sub>* together with the presence of *stx<sub>2</sub>* were the crucial points in determining the novelty of the strain just 2 days after the first stool arrived. This is of particular importance because in addition to *E coli* O104:H4, there are also other Shiga-toxin-producing *E coli* of serogroup O104, but these possess different H antigen.<sup>27</sup> The most common is Shiga-toxin-producing *E coli* O104:H21 represented in our HUSEC collection by HUSEC037. An O104:H21 strain, which is similar to the *E coli* O104:H4 outbreak strain, also *eae*-negative, caused an outbreak of bloody diarrhoea associated with consumption of raw milk in Montana, USA, in 1994.<sup>28</sup>

Taken together, the availability of the HUSEC collection including representative, well-characterised (genotypically and phenotypically) Shiga-toxin-producing *E coli* strains of all serotypes associated with haemolytic uraemic syndrome in Germany during the past 15 years and of *gnd* sequencing, *fliC*-RFLP and MLST enabled us in a very short time to identify the outbreak isolates as *E coli* O104:H4. These data and the rapidity with which the outbreak strain has been characterised show the value of standing collections of well characterised pathogens with known provenance, which was the purpose of the HUSEC collection. Moreover, implementation of well established, reproducible, and independent techniques, such as PCR and phenotypic tests available in our laboratory<sup>[4], [5], [6], [8], [12] and [22]</sup> enabled

further rapid characterisation of the outbreak strain. The outbreak strain, which is a sorbitol fermenter, would have been missed by standard diagnostic procedures focused on the O157:H7 strain and using SMAC to identify this organism on the basis of its lack of sorbitol fermentation. However, the ESBL resistance of the outbreak strain aided its recovery from patients' stool samples by use of culture on ESBL agar. This feature might be used to seek reservoirs of this strain and to recover the organism from suspected vehicles of transmission.

The reason for the apparently augmented virulence potential of the outbreak O104:H4 strain, as indicated by the high amount of haemolytic uraemic syndrome cases and 39 deaths, is unknown, but our data suggest some possibilities. The enhanced adherence of this strain to intestinal epithelial cells (figure 2) might facilitate systemic absorption of Shiga toxin and could explain the high frequency of progression to haemolytic uraemic syndrome. Antibiotic resistance might also play a part if  $\beta$ -lactam drugs used to treat the infection suppressed competing microbiota. When the outbreak strain acquired a plasmid encoding CTX-M-15, which is absent in the original HUSECO41 isolate from 2001, is unknown. This plasmid has been identified in several different genera in the Enterobacteriaceae.<sup>29</sup> Our data do not permit us to state if the Shiga-toxin-producing and enteroaggregative traits in *E coli* O104:H4 combined recently, or if this pathogen is coming to light now because of conditions suitable for its spread. An *E coli* O111 strain that expressed Shiga toxin and displayed an aggregative adherence on epithelial cells<sup>30</sup> caused an outbreak of haemolytic uraemic syndrome in France 15 years ago,<sup>31</sup> but the number of cases (ten) was lower than in the ongoing German outbreak (810 as of June 20). Differences in virulence potentials of the O111 and O104 outbreak strains, modes of transmission, the infectious dose present in the vehicle of infection (recently, sprouts have been identified as a source of the German outbreak),<sup>32</sup> and general susceptibilities of the human hosts to these two strains might play a part. Although we lack an explanation for increased virulence, this outbreak tragically shows that blended virulence profiles in enteric pathogens introduced into susceptible populations can have serious consequences for infected people.

## **Contributors**

MB and HK designed the study, analysed the data and wrote the paper. AM and RK isolated and AF serotyped the strains. AM, RK, and GP did the antimicrobial sensitivity tests. WZ developed and did the multiplex PCR for identification of *E coli* O104:H4. MB and WZ did the genomic analyses, and AB and MB the phenotypic studies. AB prepared the figures. HK and AM raised the funding. HK and GP supervised the study.

## **Conflicts of interest**

We declare that we have no conflicts of interest.

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## Tables and Figures

**Table 1.** Primers for multiplex PCR to identify *Escherichia coli* O104:H4 harbouring *stx*<sub>2</sub>

	Sequence (5'–3')	Target	Amplicon size (bp)	Primer concentration	Reference
O104rfbO-f O104rfbO-r	TGAACTGATTTTTAGGATGG AGAACCTCACTCAAATTATG	<i>rfbO<sub>104</sub></i>	351	360 nmol/L	This study
fliCH4-a fliCH4-b	GGCGAAACTGACGGCTGCTG GCACCAACAGTTACCGCCGC	<i>fliCH4</i>	201	240 nmol/L	This study
LP43 LP44	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	<i>stx</i> <sub>2</sub>	584	150 nmol/L	5
TerD1 TerD2	AGTAAAGCAGCTCCGTCAAT CCGAACAGCATGGCAGTCT	<i>terD</i>	434	240 nmol/L	6

Multiplex PCR with initial denaturation (94°C, 5 min), 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 60 s), and extension (72°C, 60 s), and final extension (72°C, 5 min).

**Table 2.** Virulence loci of 80 *Escherichia coli* O104:H4 outbreak isolates by pathogroup, compared with HUSEC041 and enteroaggregative *E. coli* strain 55989

Product		Presence in <i>E. coli</i> O104:H4*	Presence in HUSEC041	Presence in EAEC 55989†
<b>STEC</b>				
<i>stx</i> <sub>1</sub>	Shiga toxin 1	0/80	No	No
<i>stx</i> <sub>2</sub>	Shiga toxin 2	80/80	Yes	No
EHEC- <i>hly</i> <sub>E</sub> A	EHEC haemolysin	0/80	No	No
<i>cdt</i> (I-V)	Cytolethal distending toxin	0/80	No	No
<i>sub</i> AB	Subtilase cytotoxin	0/80	No	No
<i>esp</i> P	Serine protease EspP	0/80	No	No
<i>eae</i>	Intimin	0/80	No	No
<i>iha</i>	Iha (IrgA homologue adhesin)	80/80	Yes	Yes
<i>fpA</i> <sub>O26</sub>	Structural subunit of long polar fimbriae (LPF) of STEC O26	80/80	Yes	Yes
<i>fpA</i> <sub>O113</sub>	Structural subunit of LPF of STEC O113	80/80	Yes	Yes
<i>fpA</i> <sub>O167-O141</sub>	Structural subunit of LPF of STEC O157:H7 (encoded on O island 141)	0/80	No	No
<i>fpA</i> <sub>O167-O154</sub>	Structural subunit of LPF of STEC O157:H7 (encoded on O island 154)	0/80	No	No
<i>saa</i>	Saa (STEC autoagglutinating adhesin)	0/80	No	No
<i>sfp</i> A	Structural subunit of Sfp fimbriae	0/80	No	No
<i>fer</i> cluster	Tellurite resistance	80/80	Yes	No
<i>irp</i> 2	Component of iron uptake system on HPI	80/80	Yes	No
<i>fyw</i> A	Component of iron uptake system on HPI	80/80	Yes	No
<b>EAEC</b>				
<i>ast</i> A	EAEC virulence plasmid (pAA)‡	80/80	Yes	Yes
<i>agg</i> A	Pilin subunit of aggregative adherence fimbriae I (AAF/I)	80/80	No	No
<i>agg</i> 3A	Pilin subunit of aggregative adherence fimbriae III (AAF/III)	0/80	Yes	Yes
<i>agg</i> R	Transcriptional regulator AggR	80/80	Yes	Yes
<i>eap</i>	Dispersin	80/80	Yes	Yes
<i>set</i> 1	<i>Shigella</i> enterotoxin 1	80/80	Yes	Yes
<i>pic</i>	Pic (protein involved in intestinal colonisation)	80/80	Yes	Yes
<i>ast</i> A	EAEC heat-stable enterotoxin 1 (EAST1)	0/80	Yes	Yes
<b>EPEC</b>				
<i>bfp</i> A	Bundle-forming pili	0/80	No	No
<b>ETEC</b>				
<i>eht</i>	Heat-labile enterotoxin (LT)	0/80	No	No
<i>est</i> a	Heat-stable enterotoxin (STIa)	0/80	No	No
<i>est</i> b	Heat-stable enterotoxin (STIb)	0/80	No	No
<b>EIEC</b>				
<i>ial</i>	Invasive plasmid (pInv)§	0/80	No	No

STEC=Shiga toxin-producing. EPEC=enteropathogenic. EAEC=enteroaggregative. ETEC=enterotoxigenic. EIEC=enteroinvasive *E. coli*. HPI=high pathogenicity island.

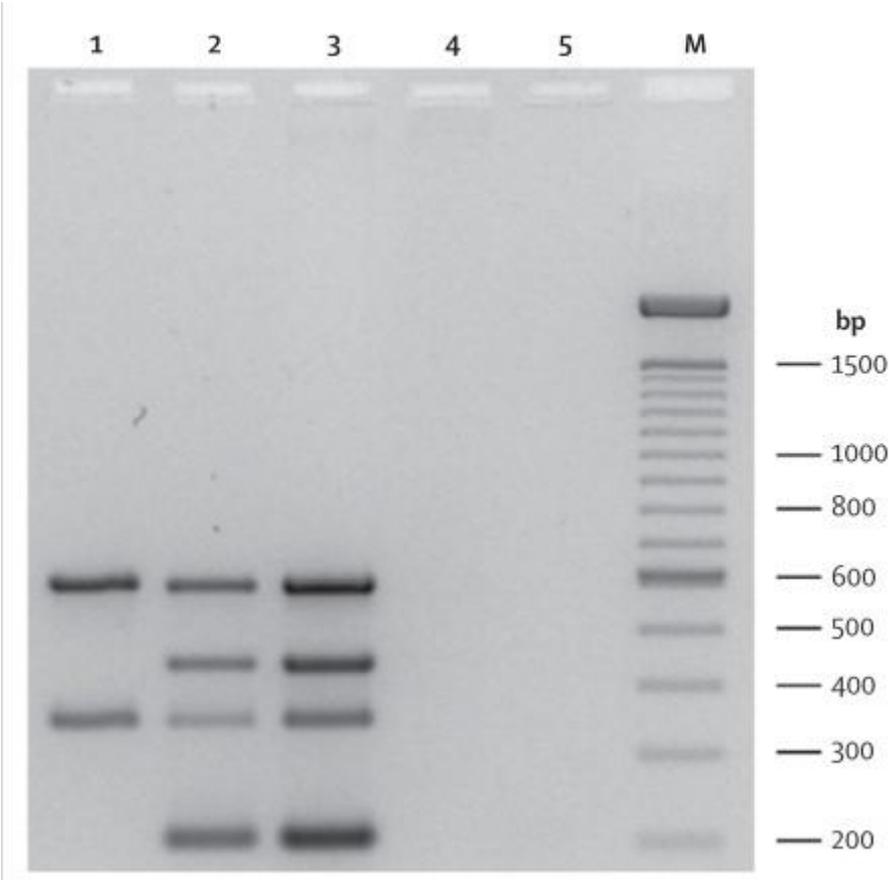
\* Number of strains positive for the locus/number tested.

† Based on in silico analysis of the sequence of EAEC strain 55989 (GenBank accession number NC\_0111748).

‡ Tested by PCR targeting the region of the EAEC virulence plasmid used as an EAEC diagnostic probe [pCVD432]<sup>19</sup> for *ast*A.<sup>20</sup>

§ Tested by PCR targeting the region of pInv used as an EIEC diagnostic probe (*ial*).<sup>21</sup>

**Figure 1.** Detection of *Escherichia coli* O104:H4 outbreak strain. The amplicons are *stx*<sub>2</sub> (584 bp), *terD* (434 bp), *rfb*<sub>O104</sub> (351 bp), *fliC*<sub>H4</sub> (201 bp). Lane 1: HUSEC037 (O104:H21; *stx*<sub>2</sub>-positive, *terD*-negative, *rfb*<sub>O104</sub>-positive, *fliC*<sub>H4</sub>-negative). Lanes 2 and 3: *E. coli* O104:H4 outbreak isolates LB226692 and LB226541, respectively. Lane 4: *E. coli* K12 negative control. Lane 5: reagent control. M: 100 kb molecular size marker.



**Figure 2.** Aggregative, so called, stacked-brick adherence to cultured intestinal epithelial cells. Outbreak *Escherichia coli* O104:H4 isolate LB226692 (A). An enteroaggregative *E. coli* strain (positive control; B). HUSEC041 (C). HUSEC037 (negative control; D).

