

Epidemiology and genomic analysis of Shiga toxin-producing *Escherichia coli* clonal complex 165 in the UK

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Abstract

Introduction. Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic, foodborne gastrointestinal pathogen that has the potential to cause severe clinical outcomes, including haemolytic uraemic syndrome (HUS). STEC-HUS is the leading cause of renal failure in children and can be fatal. Over the last decade, STEC clonal complex 165 (CC165) has emerged as a cause of STEC-HUS.

Gap Statement. There is a need to understand the pathogenicity and prevalence of this emerging STEC clonal complex in the UK, to facilitate early diagnosis, improve clinical management, and prevent and control outbreaks.

Aim. The aim of this study was to characterize CC165 through identification of virulence factors (VFs) and antimicrobial resistance (AMR) determinants in the genome and to integrate the genome data with the available epidemiological data to better understand the incidence and pathogenicity of this clonal complex in the UK.

Methodology. All isolates belonging to CC165 in the archives at the UK public health agencies were sequenced and serotyped, and the virulence gene and AMR profiles were derived from the genome using PHE bioinformatics pipelines and the Centre for Genomic Epidemiology virulence database.

Results. There were 48 CC165 isolates, of which 43 were STEC, four were enteropathogenic *E. coli* (EPEC) and one *E. coli*. STEC serotypes were predominately O80:H2 ($n=28$), and other serotypes included O45:H2 ($n=9$), O55:H9 ($n=4$), O132:H2 ($n=1$) and O180:H2 ($n=1$). All but one STEC isolate had Shiga toxin (*stx*) subtype *stx2a* or *stx2d* and 47/48 isolates had the *eae* gene encoding intimin involved in the intimate attachment of the bacteria to the human gut mucosa. We detected extra-intestinal virulence genes including those associated with iron acquisition (*iro*) and serum resistance (*iss*), indicating that this pathogen has the potential to translocate to extra-intestinal sites. Unlike other STEC clonal complexes, a high proportion of isolates (93%, 40/43) were multidrug-resistant, including resistance to aminoglycosides, beta-lactams, chloramphenicol, sulphonamides, tetracyclines and trimethoprim.

Conclusion. The clinical significance of this clonal complex should not be underestimated. Exhibiting high levels of AMR and a combination of STEC and extra-intestinal pathogenic *E. coli* (ExPEC) virulence profiles, this clonal complex is an emerging threat to public health.

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Abbreviations: AMR, antimicrobial resistance; CC, clonal complex; CGE, Centre for Genomic Epidemiology; CH, coverage and homology; EPEC, enteropathogenic *E. coli*; ESQ, enhanced surveillance questionnaire; ExPEC, extra-intestinal pathogenic *E. coli*; GBRU, Gastrointestinal Bacteria Reference Unit; HUS, haemolytic uraemic syndrome; LEE, locus of enterocyte effacement; MDR, multi-drug-resistant; MLST, multi-locus sequence typing; PHE, Public Health England; SERL, Scottish *E. coli* Reference Laboratory; ST, sequence type; STEC, shiga toxin-producing *Escherichia coli*; T3SS, type 3 secretion system; VF, virulence factor; WGS, whole genome sequencing.

The sequences derived in this study are deposited under Bioproject no. PRJNA315192, National Center for Biotechnology Information (NCBI) Short Read Archive.

One supplementary table and three supplementary figures are available with the online version of this article.

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DATA SUMMARY

FASTQ files are submitted to the National Center for Biotechnology Information (NCBI). All data can be found under Bioproject no. PRJNA315192 and strain-specific information is found in Table S1 (available in the online version of this article).

INTRODUCTION

The term haemolytic uraemic syndrome (HUS) was first used in the 1950s to describe the combination of clinical presentations of haemolytic anaemia, thrombocytopenia and renal failure [1]. Outbreaks of HUS led clinicians to suspect an infectious cause, and in 1985 Karmali *et al.* showed that Shiga toxin-producing *Escherichia coli* (STEC) could be isolated from the faecal specimens of a sub-set of HUS patients reporting a diarrhoeal prodrome [2]. Over the decades, other causes of non-infectious (atypical) HUS have been identified, but STEC remains the leading cause of HUS, especially in children [3].

Although Karmali *et al.* showed a number of different *E. coli* serotypes had the potential to produce Shiga toxin, a series of outbreaks of HUS caused by *E. coli* serotype O157:H7 during the 1980s established this serotype as the most common cause of STEC-HUS at that time, particularly in the UK and North America [4–6]. Subsequent studies showed that progression to STEC-HUS was most likely in patients infected with STEC harbouring the Shiga toxin (*stx*) type 2, in particular *stx2a* and *stx2d* [7]. The *stx* genes are bacteriophage-encoded and there is evidence that a wide range of *E. coli* serotypes other than O157:H7 have acquired the *stx2a*- (or *stx2d*) encoding phage [7].

Acquisition of *stx* by strains of *E. coli* that exhibit an attachment mechanism encoded by the locus of enterocyte effacement (LEE) result in a highly pathogenic strain capable of causing STEC-HUS [7]. The LEE encodes a Type III Secretion System (T3SS) including *espA*, *espB*, *espF*, *tir* and intimin that facilitates attachment to the human gut mucosa and microvillus effacement [8–10]. Intimin is encoded by the *eae* gene, which acts as a marker for the presence of the LEE. Non-LEE effectors include *espC*, *espI*, *espJ*, *cif*, *nleA*, *nleB* and *nleC* [8–10]. Certain STEC serotypes, including STEC O157:H7, harbour a plasmid-encoded enterohaemolysin (*ehxA*), a toxin (*toxB*) and serine protease (*espP*) [11].

Over the last 10 years, a novel serotype of STEC has emerged as a cause of STEC-HUS, STEC O80:H2 belonging to clonal complex 165 (CC165) [12–14]. This serotype has been isolated from patients with STEC-HUS presenting with multi-organ failure, extensive central nervous system involvement, and extensive thrombotic microangiopathy [12–14]. STEC O80:H2 has been described as a hybrid pathotype that combines the diarrhoeagenic *E. coli* virulence factors (VFs) *stx*, *eae* (intimin) and *ehxA* (enterohaemolysin), with extra-intestinal VFs [15].

The extra-intestinal virulence phenotype is attributed to the presence of a mosaic plasmid that harbours VFs that allow

the translocation and survival of *E. coli* from the gut to extra-intestinal sites. This mosaic plasmid is closely related to the pathogenic plasmid pS88 found in extra-intestinal pathogenic *E. coli* (ExPEC), which harbours the genes *iro* (iron acquisition), *iss* (serum resistance) and *hlyF* (haemolysin) among many others such as *sitABCD*, *iutA*, *iucC* and *ompTp* [16, 17]. This mosaic plasmid found in O80:H2 has an additional resistance cassette which confers resistance to multiple classes of antibiotics (e.g. aminoglycosides, tetracyclines and β -lactams) [15].

The aim of this study was to review the data in the national enhanced STEC surveillance systems and whole genome sequencing data held by the public health agencies in the UK to investigate the incidence, and epidemiological and microbiological characteristics of this highly pathogenic strain of STEC.

METHODS

Bacterial strains

The Gastrointestinal Bacteria Reference Unit (GBRU), Public Health England (PHE), receives faecal specimens that test positive for STEC by PCR from patients with hospital or community-acquired cases of gastrointestinal disease or HUS, in England and Wales, when diagnostic labs are unable to isolate *E. coli* O157:H7 from the stool. In Scotland, faecal specimens from patients with symptoms of bloody diarrhoea and/or HUS are referred to the Scottish *E. coli* Reference Laboratory (SERL). STEC PCR and culture are performed at GBRU and SERL as previously described [18, 19].

Data, setting and source

England, Scotland and Wales operate a national enhanced STEC surveillance system for all STEC cases encompassing epidemiological data, reported through Enhanced Surveillance Questionnaires (ESQs) and microbiological data. This study includes all samples submitted to PHE and SERL between 2013 and 2020 belonging to CC165.

For STEC patients who had completed an ESQ, we extracted results relating to travel, clinical symptoms and food/animal exposures. STEC questionnaire data were linked to whole genome sequencing (WGS) data, including *stx* subtype, virulence profiling and antimicrobial resistance (AMR) detection.

WGS using Illumina technologies and data processing

Genomic DNA belonging to isolates from CC165 was extracted and sequenced as described in Dallman *et al.* [20]. SRA accession numbers were generated and are listed in Table S1.

Variant calling, SNP typing and phylogeny from Illumina data

The population structure of CC165 was investigated using SnapperDB v0.2.6 [21], which holds the called variant data relative to a CC165 reference genome (CP002729.1) for isolates sequenced at PHE. A whole genome alignment of all samples in CC165, mapped to reference CP002729, was produced. Gubbins v2.0.0 [22] was used on all samples within

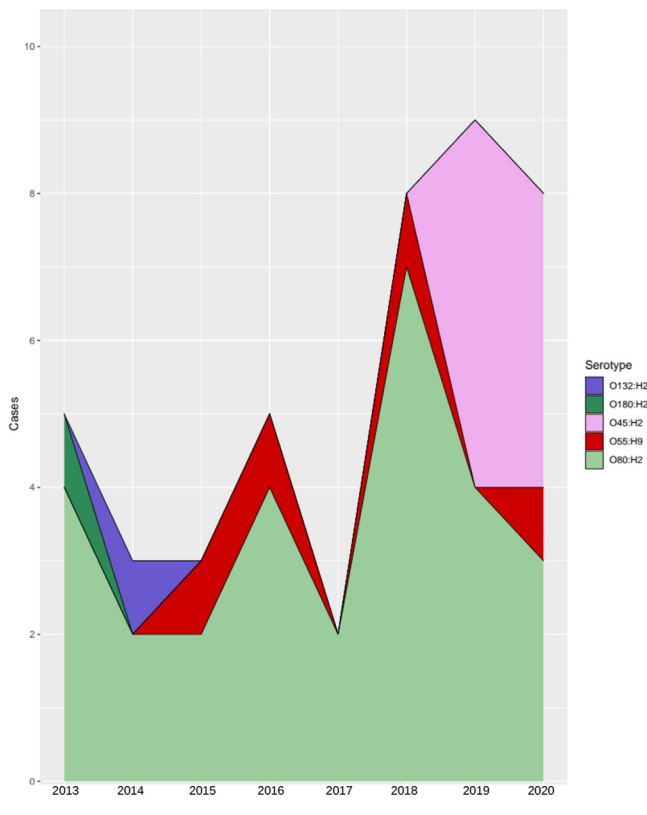


Fig. 1. Yearly distribution of CC165 STEC cases in this dataset ($n=43$). The x-axis identifies the year of the case, and the y-axis gives the cumulative cases for the year. The stacked area graph shows the different serotypes detected in each year.

the alignment and an output that identified the recombinant regions was produced. SnapperDB v0.2.6 [21] was used again to re-extract the variants belonging to CC165, producing an alignment where a given variant position belonging to a minimum of 80% of the strains in the alignment and recombination regions were masked. IQTree v2.0.4 [23] was used to construct a maximum-likelihood phylogeny using the best-fit model and this was visualized in ITOL v5.7 [24].

Enterbase (v1.1.2) was used to generate a Minimum Spanning Tree (MSTree V2) based on the Achtman 7 Gene multilocus sequence type (MLST) scheme with 10469 strains included. Data selection involved genome uploads where 'Species contains *Escherichia coli*' and 'Lab contact contains Public Health England or GBRU' between 2014 and 2021.

Virulence and AMR profiling using Illumina genome data

Virulence profile and AMR profiles were derived from the Illumina genome data as previously described [25–27], using the GeneFinder tool (https://github.com/phe-bioinformatics/gene_finder). The presence of each gene was confirmed when coverage and homology (CH) are over 85% compared to the reference gene. For the purpose of this study, genes where CH fell below 85% when compared to the reference were

investigated further. The PHE virulence database was supplemented with variants of putative genes in *E. coli* virulence database v2020-05-29 from the Centre for Genomic Epidemiology (CGE) (https://bitbucket.org/genomicpidemiology/virulencefinder_db/src/master/virulence_ecoli.fsa).

Plasmid identification

The pO157 plasmid sequence (Accession AF074613.1) [28], as well as plasmid sequences corresponding to pR444_A and pR444_C (QBDM01000004.1 and QBDM01000002.1, ASM312350v1, BioProject PRJNA449634) [15] were extracted and curated into a custom database, designed for SRST2 [29]. SRST2 v0.2.0 was used to identify the presence of pR444_A, pR444_C and similarity to pO157 using Illumina FASTQ, with parameters for minimum coverage set at 40 and to report all consensus sequences.

Data deposition

Illumina FASTQ files are available from the National Center for Biotechnology Information (NCBI) Short Read Archive Bioproject no. PRJNA315192. The SRA (sequence read archive) accession numbers are given in Table S1.

RESULTS

Epidemiology

Since 2013, 43 isolates of STEC belonging to CC165 were detected in England ($n=34$), Scotland ($n=5$) and Wales ($n=4$). The majority of cases were female (26/43, 60%) and the age range was 0–85 years, with 30% (13/43) of cases under 5 years. Cases were reported throughout the year, with the highest number of cases reported in 2019 (Fig. 1). The maximum, minimum and median cases reported across the years for CC165 STEC were nine, three and five respectively. Geographical distribution in England revealed that London had the most cases ($n=13$), followed by the South East region ($n=10$) (Fig. S1). The remaining cases were distributed across the rest of the PHE centres, not surpassing three cases per PHE centre.

Of the 34 cases resident in England, an enhanced questionnaire was available for 32 cases. Fewer than half of the cases infected with CC165 reported either vomiting, nausea, abdominal pain and/or fever (Table 1). The majority of cases had diarrhoea (30/32, 94%) but only 5/32 (16%) reported bloody diarrhoea, compared to cases infected with STEC O157:H7 where 93–95% had diarrhoea and 61–62% had bloody diarrhoea (PHE in-house data). There were 13/32 (41%) hospitalized cases, including 8/32 cases diagnosed with HUS (25%), of which half were female (4/8, 50%) and 6/8 (75%) were aged between 0 and 4 years. The two adults were 63 and 85 years old. Compared to STEC O157:H7, cases of CC165 STEC had a higher rate of hospitalisation (CC165: 41% vs O157: 34%) and progression to STEC-HUS (CC165: 25% vs O157: 3–5%) (PHE in-house data).

ESQ data were available for none of the Welsh cases, and for 3/5 Scottish STEC cases. Of the three cases with extended

Table 1. Clinical presentation ($n=32$); England STEC cases where an ESQ was available

Symptom	N	%
Diarrhoea	30	94
Abdominal pain	16	50
Vomiting	14	44
Hospitalized	13	41
Fever	12	38
Nausea	10	31
HUS	8	25
Blood in stools	5	16
Death	0	0

data, 3/3 (100%) presented with bloody diarrhoea, 2/3 (67%) reported abdominal pain, and 1/3 (34%) reported nausea and vomiting. No cases were hospitalized overnight, but one case attended as a day case.

None of the cases resident in Scotland (0/3) reported travelling outside the UK. There were 10/32 England cases who reported having travelled outside of the UK, of which seven cases travelled to France, two cases reported travelling to Portugal and one travelled to Egypt.

ESQs for data from England revealed that 11/32 (34%) cases reported contact with dogs and 6/32 (19%) reported contact with cats. One case was reported for contact with each of the following: cattle, horses, poultry and deer. Three of 32 cases (9%) visited a farm, 4/32 cases (19%) walked on a farm where animals graze, 3/32 (9%) had contact with wildlife (bird feeder, possible deer droppings, pigeons or cats) and 4/32 (13%) had contact with soil, manure or sewage (Table 2). Food history of cases saw the most common food exposure was cooked poultry (18/32, 56% of cases), with raw vegetables following at 16/32 cases (50%) (Table 2).

Scottish enhanced surveillance data revealed that contact with cattle and/or cats was reported by three cases. Two of the three cases live on a farm. There were no common food exposures.

Phylogenetic analysis

We investigated the population structure (based on seven-gene MLST) of *E. coli* submitted to Enterobase by PHE between January 2014 and January 2021, highlighting the position of CC165 in the *E. coli* population (Fig. S2). CC165 in this data set comprises sequence type (ST) 301, ST165, ST189, ST382, ST1178 and ST6312.

There were 48 isolates belonging to CC165 in the PHE archive belonging to England, Wales and Scotland, comprising ST301 ($n=43$), ST189 ($n=3$), ST382 ($n=1$) and ST165 ($n=1$) (Table S1). The isolate belonging to ST165 was negative for both *eae* and *stx*. There were four isolates that had *eae* but were *stx*-negative and were designated enteropathogenic *E. coli*

Table 2. Environmental and food exposures ($n=32$); England STEC cases where an ESQ was available

Environmental/food exposure	N	%
Dog	11	34
Cat	6	19
Cattle	1	3
Horse	1	3
Poultry	1	3
Deer	1	3
Farm where animals graze	6	19
Soil, manure or sewage	4	13
Wildlife	3	9
Farm	3	9
Cooked poultry	18	56
Raw vegetables	16	50
Pasteurized milk	17	53
Hard cheese	15	47
Yoghurt	14	44
Fish	13	41
Cooked beef	13	31
Ice cream	8	25
Cooked pork	7	22
Soft cheese	7	22
Raw beef	5	16
Cured meats	5	16
Raw poultry	4	13

(EPEC). The isolates of EPEC belonged to ST189 ($n=3$) and ST382 ($n=1$). A phylogeny of CC165 was built with CP002729 as the reference. Three samples were excluded from the phylogeny by SnapperDB due to the mean depth coverage being below the cut-off threshold of 30. This resulted in 46 leaves in the tree, including the reference which was generated by aligning simulated reads to itself (Fig. 2). The sample belonging to ST382 (O unidentifiable:H5) was excluded from the phylogeny for the reasons stated, and therefore EPEC ST189 (O157:H26, $n=3$) and *E. coli* ST165 (O80:H19, $n=1$) belonged to the cluster designated as Cluster (C)1.

C2 comprised the isolates that harbour the *stx*-encoding prophage (STEC) and was divided into two sub-clusters, ST301C1 and ST301C2. ST301C1 comprised isolates belonging to serotype O80:H2 ($n=18$), whereas ST301C2 contained isolates belonging to three different serotypes, O55:H9 ($n=4$), O45:H2 ($n=8$) and O80:H2 ($n=9$). Two isolates of ST301 (serotypes O180:H2 and O132:H2) did not cluster with ST301C1 or ST301C2. Fig. 1 also describes the

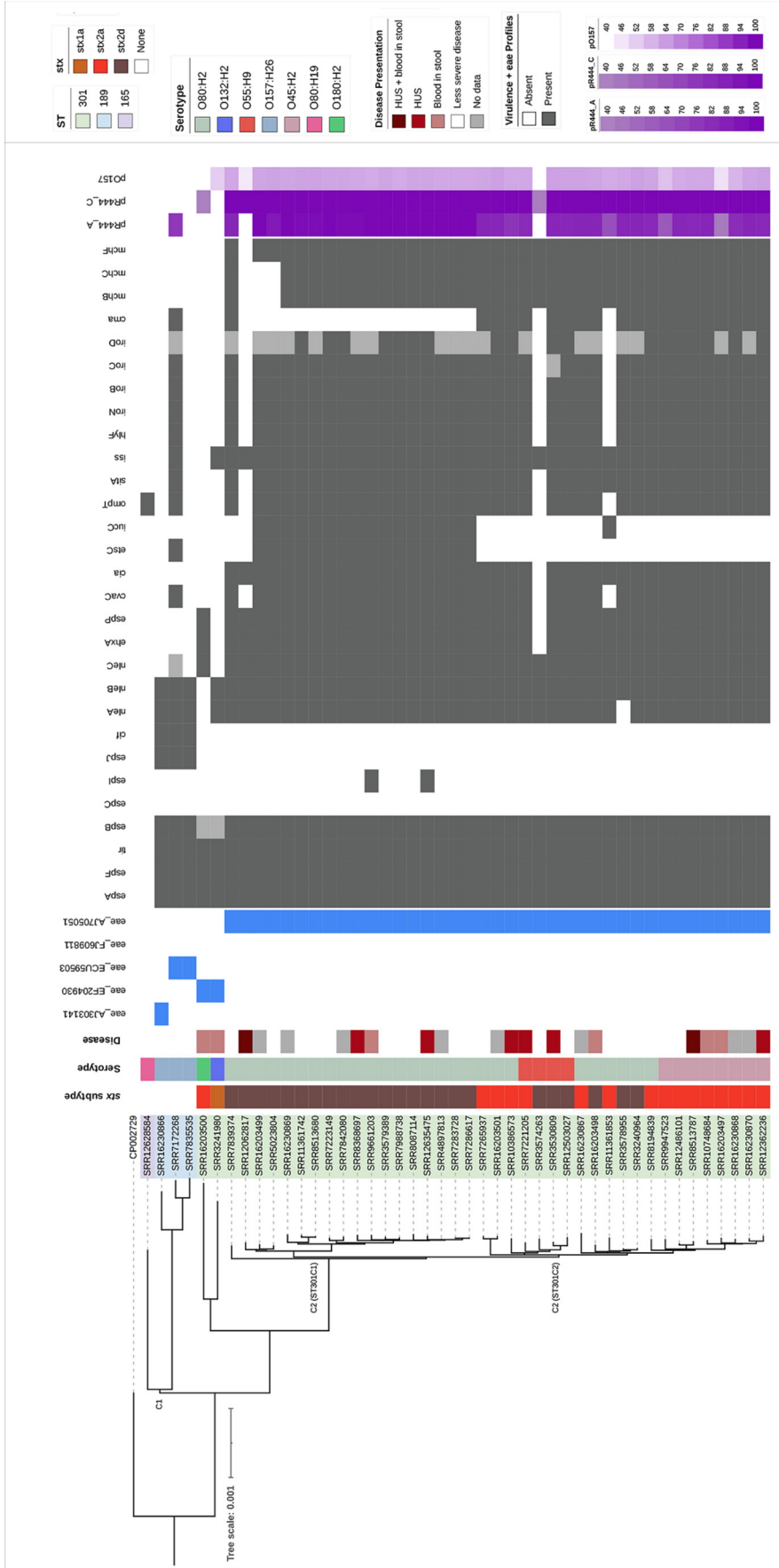


Fig. 2. Population structure of CC165 in the UK (n=46) (three isolates excluded), constructed using SnapperDB and IQTree, and rooted at the midpoint. Sequence type, stx subtypes, severe clinical diagnosis and serotype are the first four colour strips mapped onto the phylogeny. The presence of variants of the eae gene is shown as blue for detected and white for not detected. VF profiles achieved from PHE pipelines supplemented with genes from CGE are annotated on the population structure of CC165 in the UK. Dark grey indicates detection, whereas light grey indicates the gene was not detected but coverage and homology values are high (>60). Purple on the right of the VF annotation indicates identity coverage of isolates to the three plasmid references, pR444_A, pR444_C and pO157. Shades of purple indicate coverage identity values, with light purple being 40.

emergence of CC165 STEC serotypes from 2013 to 2020, with O45:H2 first emerging into the population in 2019.

STEC virulence factors

As described by Blanco *et al.*, and Cointe *et al.* [15, 30], isolates of O80:H2 are characterized by a unique variant of the *eae* gene, *eaeXi* (AJ705051). Isolates of CC165 were examined for *eae*, using variants from the CGE database. There were 41 isolates of CC165 that harboured the *eaeXi* variant, which were all isolates in C2, and two isolates that were excluded from the tree, which includes serotypes O80:H2, O55:H9 and O45:H2. The remaining six isolates that had *eae* detected harboured four other variants (Fig. 2).

There were 43 isolates, all ST301, that harboured *stx2d* ($n=25$), *stx2a* ($n=17$) and *stx1a* ($n=1$) (Table S1). Isolates that were positive for *stx2a* belonged to four serotypes: O45:H2 ($n=9$), O80:H2 ($n=6$), O55:H9 ($n=1$) and O180:H2 ($n=1$). Isolates that harboured *stx2d* belonged to two different serotypes: O80:H2 ($n=22$) and O55:H9 ($n=3$) (Table S1). Of the eight cases that were diagnosed with HUS, four were infected with isolates that had *stx2a* and four had *stx2d*.

Fig. 2 shows the distribution of VFs for each isolate across the population structure of CC165.

As expected, LEE-encoded T3SS proteins (*espA*, *espB*, *espF* and *tir*) were present in all *eae*-positive isolates [8, 31]. We also interrogated the genomes to determine whether non-LEE-encoded effectors, *espC*, *espI*, *espJ*, *cif*, *nleA*, *nleB* and *nleC*, were present [10, 32, 33]. For the majority of samples, *nleA* and *nleB* were present in the *eae*-positive isolates whereas *nleC* was only confirmed in isolates with *stx2a* or *stx2d*. Using the 85% coverage and homology criteria, *espC* was not detected in any of the isolates of CC165, but CH values <10% for all but one isolate were detected (ST2). The gene *espC* is not considered essential in attaching and effacing lesion formation [32]. The presence of *espI* was confirmed in two STEC isolates, although lower CH values for *espI* were detected for other isolates (ST2). The genes *espJ* and *cif* were detected in 3/4 EPEC isolates, but not in those that had acquired *stx* (Fig. 2).

In STEC O157:H7, the pO157 plasmid harbours *toxB*, *katP*, *ehxA* and *espP* [11, 28, 34–37]. In our dataset, both *ehxA* (an enterohaemolysin) and *espP* (an extracellular serine protease associated with the LEE [35]) were detected in all STEC isolates that harboured *stx2a* or *stx2d*, with one exception ($n=41$). The gene *toxB* was detected in one EPEC isolate (ST2) and *katP* was not detected in any of the isolates in this dataset.

VFs associated with the pO157 plasmid were present in our dataset, including the genes *ehxA* and *espP*. However, coverage against the pO157 reference was between 45 and 64 % in 41 STEC isolates and one EPEC (Fig. 2). Our analysis also included the pR444_C 95 kb plasmid identified by Cointe *et al.* [15]. The results did not reveal much variation between STEC clusters, with coverage ranging between 99.5 and 100% with two exceptions, in relation to the reference pR444_C (Fig. 2).

Extra-intestinal VFs

The plasmid-encoded haemolysin, *hlyF*, is located on the pS88-like plasmid [15, 17, 38] and was detected in most STEC (38/43) and one EPEC (1/4) (total=39/48). Other genes encoded on the pS88-like plasmid, as described by Cointe *et al.* [15, 17], were investigated. The genes *cvaC*, *cia*, *ompT* and *sitA* appear to be present in both ST301C1 and ST301C2 (Fig. 2). Conversely, *etsC* and *iucC* were present mostly in ST301C1 (Fig. 2). All but one STEC isolate ($n=42$) and one EPEC isolate had *iss* (43/48). The gene *iss* is a serum resistance-associated protein and is also encoded on the pS88 or pS88-like plasmid [17, 39].

The *iro* gene cluster is responsible for extra-intestinal iron aggregation commonly associated with ExPEC and the genes are encoded on a plasmid [17, 40–42]. The genes *iroB*, *iroC* and *iroN* were detected in most STEC isolates as well as one EPEC (Fig. 2). The presence of *iroD* was lower, with some STEC isolates below the threshold for detection (Fig. 2, ST2).

The genes *cma*, *mchB*, *mchC* and *mchF* are associated with microcin, a natural bactericidal antibiotic, with *cma* suggested to be encoded on a plasmid [15, 38, 43, 44]. The genes *mchB*, *mchC* and *mchF* were detected in all isolates that belonged to ST301C2. In ST301C1, *mchB* and *mchC* were present in 15/18 isolates while *mchF* was present in 17/18. (Fig. 2). The gene *cma* was detected in 23/48 isolates, mainly ST301C2 (19/21) and four isolates outside the cluster including one EPEC (Fig. 2, ST2).

The presence of a pS88-like plasmid (pR444_A [15]) across CC165 was investigated, with genes that are encoded on the pS88 plasmid being found in most STEC isolates. The majority of ST301C1 had over 90% coverage for a pS88-like plasmid. Variation against the reference pR444_A in ST301C2 was detected, with C2 coverage ranging from 44 to 86% (Fig. 2). The coverage correlating to this plasmid, as well as the presence of genes that are typically encoded on the pS88 plasmid suggest the presence of a pS88-like plasmid in STEC isolates and one EPEC (Fig. 2).

Antimicrobial resistance

Eighty-eight per cent (42/48) of isolates in CC165 were multidrug-resistant (MDR), defined as resistant to three or more antimicrobial classes (Fig. 3). Phenotypic AMR profiling was not conducted, as studies have previously assessed the relationship of genotype and phenotype in non-O157 STEC isolates [27].

Thirty-nine of 48 (81%) isolates had AMR determinants known to confer resistance to beta-lactams, with 87% (34/39) isolates harbouring $\text{bla}_{\text{TEM-1}}$. Forty-two of 48 (88%) isolates had a mutation at position 83[S-L] in *gyrA*, known to confer reduced susceptibility to ciprofloxacin [27] (fluoroquinolones).

Resistance to aminoglycoside was determined by the presence of *strA-strB*, *ant2'-Ia*, *aph6-Id* or *aad* variants; 32/48 isolates (67%) held one or more of these determinants. Trimethoprim resistance was present in 40/48 isolates, with *dfrA36* being

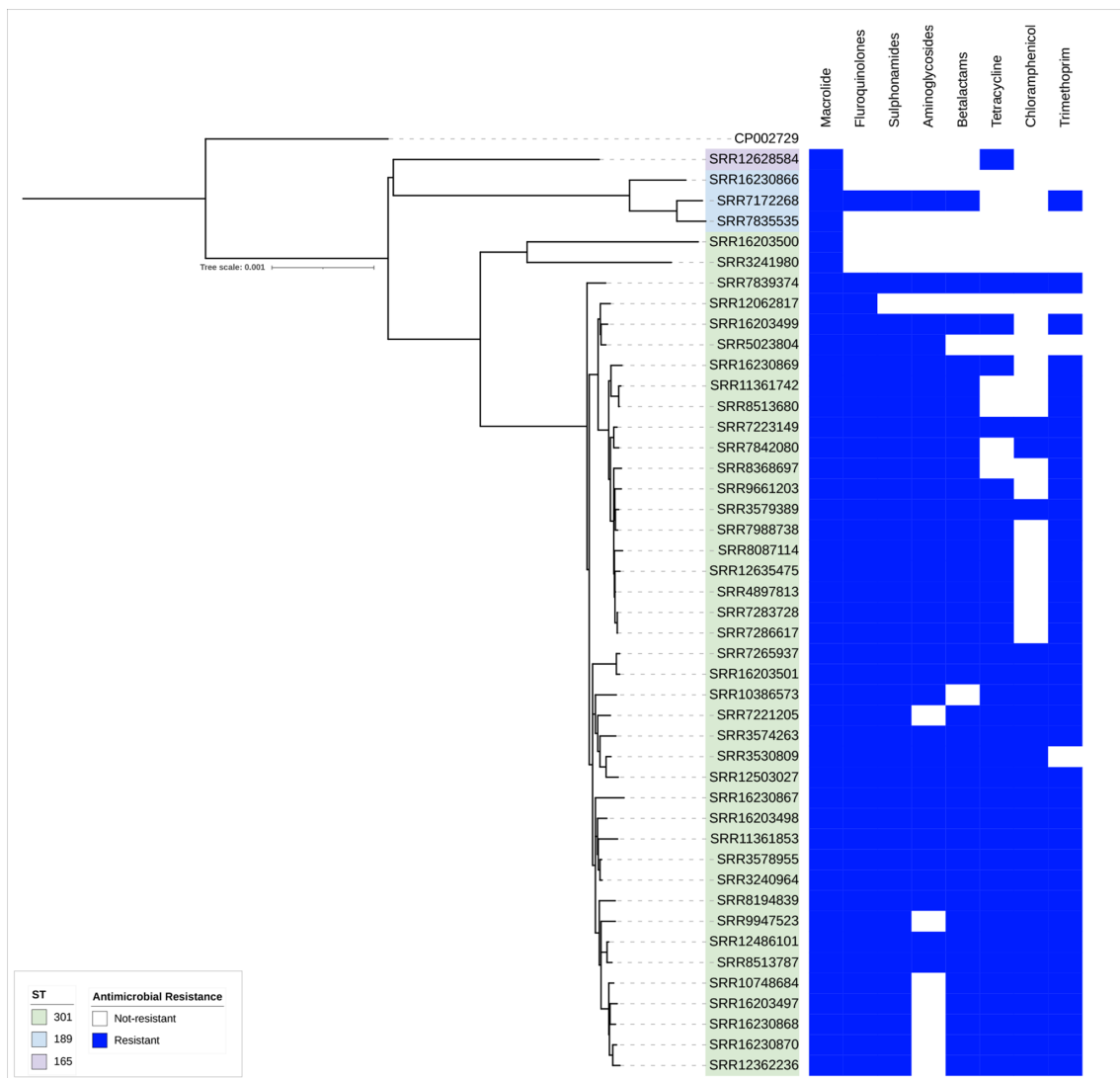


Fig. 3. AMR profiles achieved from the PHE pipeline supplemented with trimethoprim genes *dfrA34*, *dfrA35* and *dfrA36* from CGE, annotated on the phylogeny of CC165 in the UK. The blue box indicates a resistance gene was detected. In the case of fluoroquinolone, the blue box indicates reduced susceptibility.

most common ($n=25/40$, 63%), followed by *dfrA-5* ($n=15$). We noted a striking difference in gene presence between ST301C1 and ST301C2, with C2 predominantly harbouring *dfrA36*.

There were 36/48 isolates with *tetA*, with one isolate also harbouring *tetM*. Forty-one of 48 isolates had *sul-1*, *sul-2* or both, with one isolate also having *sul-3*. Chloramphenicol resistance was held by 27/48 isolates, attributed to *catA-1* ($n=3$), *floR* ($n=9$), *catA-1* and *floR* ($n=14$), and *catA-1*, *floR* and *cml-1* ($n=1$).

Isolate SRR3574263 that had *sul1*, 2 and 3 also had *bla*_{CTX-M-32}, *tetA* and *tetM*, the three chloramphenicol genes, *dfrA-1* and *dfrA36*, macrolide resistance *ermB*, the gyrase mutation and *aadA-23*, *aph6-Id*, *strA-strB*, *aadA-3*, *aac3-IIa* and *ant2''-Ia*,

a total of 20 AMR determinants, from the eight antibiotic classes investigated.

DISCUSSION

Although overall the number of cases remains low compared to those infected with STEC O157:H7 (CC11) or STEC O26:H11 (CC29), here we present evidence that strains of STEC belonging to CC165 are emerging as a cause of STEC-HUS in the UK. While this complex is not present at high levels in the UK population, we did see higher levels in certain regions. The majority of cases occurred in London and the South East of England, although this is probably due the higher number of hospitals using commercial PCR for the detection of gastrointestinal pathogens in this region.

CC11 and CC29 are dominated by one serotype, STEC O157:H7 and STEC O26:H11, respectively, whereas CC165 is composed of at least four different STEC serotypes. While STEC O80:H2 was the dominant serotype within CC165, STEC O45:H2, STEC O55:H9, STEC O132:H2 and STEC O180:H2 were also reported in this complex in the UK.

As previously described in other countries, the hospitalization rate and proportion of cases developing HUS in the UK was higher for cases infected with STEC belonging to CC165 compared to STEC O157:H7, although the rate of bloody diarrhoea was lower in CC165 STEC. Symptoms of CC165 STEC that are comparable to STEC O157:H7 were diarrhoea, nausea, abdominal pain and vomiting.

Additionally, the diagnosis of HUS was not exclusive to the O80:H2 serotype, with the serotypes O55:H9 and O45:H2 being associated with the progression of HUS in this dataset. A lower proportion of cases reported bloody diarrhoea compared to cases of STEC O157:H7 and this may confound diagnosis and be a contributing factor in the atypical STEC-HUS presentation described by colleagues elsewhere [13].

With so few cases in the UK to date, it is difficult to determine whether strains belonging to CC165 have become endemic in the UK, or whether they are associated with travellers' diarrhoea, imported food products or animal migration. Recent data suggest that serogroup O80:H2 has become the second most frequent STEC serogroup isolated in France, after STEC O157:H7 [45, 46], and a subset of UK cases reported travelling to France prior to the onset of symptoms. Similar to this dataset, STEC isolates belonging to CC165, such as serotype O80:H2, O55:H9 and O45:H2 that harbour extra-intestinal virulence factors, have caused gastrointestinal symptoms in humans in Switzerland [47, 48], the Netherlands [13, 49] and Italy [49], supporting the conclusion that this hybrid pathotype is not limited to one serotype.

Analysis of recent exposures from animal contact and outdoor activities did not reveal any common exposures, but there was reported contact with animals and farmland, which has also been reported in STEC O157:H7 over the past 10 years. STEC O80:H2 has been isolated from cattle in Spain [30] and Belgium [16]. These data suggest that like STEC O157:H7 and STEC O26:H11, CC165 STEC has a zoonotic reservoir, and is likely to be transmitted to humans via the food chain and/or contact with animals. To date, there have been very few cattle surveys screening for non-O157 STEC in the UK. Monitoring the prevalence of CC165 STEC along with other non-O157 in cattle and other ruminants would facilitate our understanding of the source and route of transmission in the UK. The prevalence and severity of this serogroup make it essential to establish effective typing technology used for monitoring the transmission of CC165 STEC strains throughout the food chain and environment.

Unusually for STEC, isolates belonging to CC165 are characteristically MDR with the majority of isolates exhibiting resistance to eight different classes of antibiotic [27, 50]. Although antibiotic treatment of STEC is contraindicated

because certain antibiotics may induce toxin release, which enhances the risk of progression to HUS, patients are often treated when they first present to healthcare centres because the clinical picture often mimics sepsis (diarrhoea, confusion, hypotension, leukocytosis and acute renal failure). Moreover, there is an argument that once HUS has developed, antimicrobial therapy may shorten the course of illness and improve clinical outcomes [51, 52].

In 2020, a study by Gentle *et al.*, [27] investigated the correlation between genotypic prediction and phenotypic expression of AMR in STEC. The authors noted a discrepancy between isolates of CC165 that were phenotypically resistant to trimethoprim but had no AMR genes known to confer resistance to trimethoprim. These isolates were included in this study and we detected *dfrA36*. This gene, known to confer resistance to trimethoprim, was first characterized by Wüthrich *et al.* [53] in 2019, but it first appeared in this dataset in 2013 and has been circulating in the population since.

Azithromycin is an antimicrobial used for STEC treatment when appropriate, but the high levels of macrolide resistance within this dataset suggest this treatment would not be effective. The high levels of resistance in CC165 are concerning, particularly relating to the emergence of previously undetected genes, and should treatment be deemed appropriate for STEC infections, it is important that clinicians are aware of the MDR profile of strains belonging to CC165.

The majority of UK isolates in CC165 belonged to STEC O80:H2 and, as described in previous studies in France, Spain and the Netherlands, had *stx2d* and *eae*. Strains of STEC harbouring both *stx2d* and *eae* are rare in the UK but have been associated with severe clinical outcomes, including HUS, elsewhere [54, 55]. STEC O80:H2 has been described as causing severe extra-renal manifestations such as protracted seizures, myocardial damage, transaminitis, bacteraemia and peritonitis [12–14]. The rate of hospitalization in cases in England from this study was 40%, with 25% of cases having complications of HUS, but the drawback of this study is that there was lack of detailed clinical data and outcomes. We also detected STEC O80:H2 harbouring both *stx2a* and *eae*, which is the virulence gene combination most commonly seen in non-O157 STEC-causing HUS in the UK (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/732577/non-O157_STEC_Evidence_Base.pdf). Previous studies have shown that the presence of *stx2a* and *stx2d* is significantly associated with severe clinical manifestations [56].

Like STEC O157:H7 and STEC O26:H11, two other STEC serotypes that cause HUS, isolates in this study also had a combination of non-LEE-encoding effectors and *ehxA*. In contrast to other STEC serotypes, they also carried a suite of virulence factors encoded on a pS88-like plasmid more commonly found in extra-intestinal *E. coli* that may facilitate survival outside the gut. The pO157 plasmid was not found in our isolates, although two VFs (*ehxA* and *espP*) characteristic of pO157 were detected, demonstrating the dynamic

nature of genes on mobile genetic elements. By integrating the virulence gene profile with the phylogeny, we observed loss and gain of genes within the CC165 population. Specifically, acquisition of the *stx*-encoding phage corresponded to the loss of phage-encoded *espJ* and *cif*, where three of the four EPEC harboured *espJ* and *cif*. We also observed the loss or gain of individual plasmid-encoded genes, rather than the loss of the entire plasmid. We theorize that this is due to either two separate acquisition events of two highly similar plasmids into the STEC cluster, or a selective pressure from the environment that induced gene loss between the STEC subclusters, ST301C1 and ST301C2.

Here, we present the emergence of this new clonal complex in the UK. There is evidence of domestically acquired infection as the majority of cases do not report travel outside the UK, but it is not clear whether the source is imported food or whether STEC belonging to CC165 is endemic in the UK cattle population. There is also evidence of the pathogenic potential of this clonal complex and that clinical management may be challenging due to the complex presentation of patients and the multidrug resistance exhibited by the pathogen. The lack of comprehensive microbiological and/or epidemiological surveillance of STEC other than serotype O157:H7 in the UK continues to be a concern. There is a need to expand the implementation of methods capable of detecting all STEC serotypes, specifically PCR targeting *stx* at the local hospital level. Studies focusing on the detection of STEC O157:H7 from food and animal samples should be extended to include all STEC to better understand the zoonotic reservoir and transmission routes of this pathogen.

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Author contributions

Conceptualization: T.J.D., C.J. Methodology: E.V.R., T.J.D., C.J. Software: E.V.R., T.J.D. Validation: E.V.R. Formal Analysis: E.V.R., B.V. Investigation: E.V.R. Resources: R.S., L.B., A.S.P., L.A., A.H., T.J.D., C.J. Data Curation: T.J.D., C.J. Writing – Original Draft Preparation: E.V.R., C.J. Writing – Review and Editing: E.V.R., B.V., R.S., L.B., A.S.P., L.A., A.H., L.B., G.G., N.M., T.J.D., C.J. Visualization: E.V.R., B.V. Supervision: G.G., L.B., N.M., T.J.D., C.J. Project Administration: N.M., T.J.D., C.J. Funding: N.M., T.J.D., C.J.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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