

1 **Characterisation of an emergent clone of Enteroinvasive *Escherichia coli* circulating**
2 **in Europe**

3
4 **Running title:** An emerging EIEC clone causing infections in Europe

5 **Keywords:** Enteroinvasive *Escherichia coli*, *Shigella*, outbreaks of infections, genomic
6 characterisation, emergence of new pathogenic types

7 **Category:** Original Article – E-only

8
9 *Michelacci V.^{1#}, Prosseda G.², Maugliani A.¹, Tozzoli R.¹, Sanchez S.³, Herrera-León S.³,*
10 *Dallman T⁴, Jenkins C.⁴, Caprioli A.¹ and Morabito S.¹*

11
12 ¹*European Union Reference Laboratory for Escherichia coli, Department of Veterinary Public Health and*
13 *Food Safety, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161, Rome Italy*

14 ²*Istituto Pasteur-Fondazione Cenci Bolognetti, Department of Biology and Biotechnology “C. Darwin”,*
15 *Sapienza Università di Roma, Roma, Italy,*

16 ³*Laboratory of Enterobacteriaceae, Service of Bacteriology, National Center of Microbiology, Instituto de*
17 *Salud Carlos III, 28220, Majadahonda, Madrid, Spain*

18 ⁴*Gastrointestinal Bacteria Reference Unit, Public Health England, 61 Colindale Ave, London NW9 5HT, UK*

19
20 **#Corresponding author. Mailing address:**

21 European Union Reference Laboratory for *Escherichia coli*

22 Department of Veterinary Public Health and Food Safety

23 Istituto Superiore di Sanità

24 Viale Regina Elena 299, 00161, Rome, Italy

25 Tel. +39-06-49902729; Fax +36-06-49387077

26 E-mail valeria.michelacci@iss.it

27

28 **Abstract**

29 Enteroinvasive *Escherichia coli* (EIEC) cause intestinal illness indistinguishable from that
30 caused by *Shigella*, mainly in developing countries. Recently an upsurge of cases of EIEC
31 infections has been observed in Europe, with two large outbreaks occurring in Italy and in
32 the United Kingdom. We have characterised phenotypically and genotypically the strains
33 responsible for these epidemics together with an additional isolate from a sporadic case
34 isolated in Spain. The three isolates belonged to the same rare serotype O96:H19 and
35 were of sequence type ST-99, never reported before in EIEC or *Shigella*. The EIEC strains
36 investigated possessed all the virulence genes harboured on the large plasmid conferring
37 the invasive phenotype to EIEC and *Shigella*, while showing only some of the known
38 chromosomal virulence genes and none of the described pathoadaptative mutations. At
39 the same time, they displayed motility abilities and biochemical requirements resembling
40 more closely those of the non-pathogenic *E. coli* rather than the EIEC and *Shigella* strains
41 used as reference.

42 Our observations suggested that O96:H19 strains belong to an emerging EIEC clone,
43 which could be the result of a recent event of acquisition of the invasion plasmid by
44 commensal *E. coli*.

45

46 **Introduction**

47 Enteroinvasive *Escherichia coli* (EIEC) are a group of pathogenic bacteria causing
48 intestinal illness upon invasion of the human colonic mucosa [1]. The disease caused by
49 EIEC is a bacillary dysentery with a clinical presentation indistinguishable from that caused
50 by infection with strains of *Shigella* species, involving abdominal cramps, nausea, fever
51 and bloody and mucus diarrhoea [2]. The pathogenesis of EIEC infection involves the
52 colonic epithelial cell penetration preceded by the transcytosis through M cells, the lysis of

53 the endocytic vacuole, intracellular multiplication and extension into adjacent epithelial
54 cells [1].

55 The main genes conferring the *Shigella* and EIEC invasive phenotype are harboured on a
56 large plasmid and encode the components of a type three secretion system, including *mxl*
57 (Membrane excretion of Ipa) and *spa* (Surface Presentation of invasion plasmid Antigens)
58 and a number of translocated effectors, represented by the products of the genes *vir*, *ipa*
59 (Invasion Plasmid Antigens) and *ipg* (Invasion Plasmid Genes) [3]. Several other virulence
60 genes play accessory roles in the pathogenetic process and are differentially distributed in
61 different *Shigella* and EIEC strains, and encode toxins, proteins interfering with the
62 immune response of the host, factors facilitating the colonization process and iron-uptake
63 systems favouring intracellular growth [3].

64 The morbidity and mortality of EIEC infections have not been fully assessed, but can be
65 inferred from those ascribed to shigellosis. Mortality is especially high among children and
66 it has been estimated that 99% of the appraised 165 million cases recorded annually
67 worldwide occur in developing countries [4, 5]. The high circulation of these pathogens in
68 low-income regions is plausibly linked to the mode of transmission of the infections, which
69 involves the oral-faecal route. In the United States and Europe, where higher hygiene
70 standards are in place, the subjects most often infected are travellers returning from high
71 incidence countries, children in day care and migrant workers [6]. The information on the
72 incidence of EIEC-associated disease is scanty, as the differentiation between these
73 infections and those caused by *Shigella* is difficult and based on the use of multiple tests,
74 such as the PCR targeting the *ipaH* gene, coupled with biochemical and serological typing
75 [2]. In some cases the infections caused by *Shigella* and EIEC may be transmitted by
76 contaminated food and water, but these appear not to be common sources of infections
77 [7]. Historically, EIEC have not been associated with large outbreaks in industrialised
78 countries. More commonly, EIEC causes sporadic cases afflicting specific risk groups.

79 However, recently an upsurge of cases of EIEC infections has been observed in Europe.
80 In 2012, a large outbreak of bloody diarrhoea occurred among the employees of the Fire
81 Brigade of the city of Milan, Italy [8]. The episode involved more than 100 cases of
82 infections and the additional symptoms most commonly reported were fever, abdominal
83 cramps and vomiting [8]. Laboratory investigations showed the presence of a positive PCR
84 amplification of the *ipaH* gene in several stool samples and an *E. coli* strain positive for the
85 presence of *ipaH* and belonging to serotype O96:H19 was isolated from six cases [8].
86 Cooked vegetables were suspected as the source for infection following a case-control
87 study [8]. In 2013, an EIEC isolate of the same serotype was isolated from a sporadic case
88 of traveller's diarrhoea in Spain (data not published). Finally, an outbreak of
89 gastrointestinal disease occurred in the East Midlands in the United Kingdom (UK),
90 involving 50 people and was suspected to be caused by the consumption of contaminated
91 salad vegetables [9]. Again, an EIEC of serogroup O96 was isolated from some of the
92 patients [9].

93 In this paper, we carried out the biochemical and phenotypic characterisation of the EIEC
94 strains from the Italian and the UK outbreaks and from the sporadic case in Spain, as well
95 as their whole genome sequencing. Our results show that these isolates belong to a same,
96 emerging EIEC clone.

97

98 **Materials and methods**

99 **Bacterial strains**

100 The EIEC isolates involved in the study included the six strains EF432, EF433 and EF434
101 isolated in Italy in 2012 [8], H142690012 isolated in the United Kingdom 2014 [9] and
102 CNM-2113/13, isolated from a case of severe diarrhoea occurred in Spain in 2014 (data
103 not published). All the six EIEC strains possessed the *ipaH* gene, which is the hallmark for
104 EIEC as well as for *Shigella* spp strains, as assessed by conventional PCR [10]. The strain

105 EF432 was chosen as representative of the clone that caused the Italian outbreak of
106 infections and used in all the characterisation experiments, while the remaining two Italian
107 isolates (EF433 and EF434) were only included in the PFGE cluster analysis.

108 The reference EIEC strains 6.81 and 4608 [11], the *Shigella flexneri* strain M90T [11], the
109 *E. coli* K12 strain MG1655 [12] and the non-pathogenic human *E. coli* isolate ECOR1 part
110 of the ECOR reference collection [13] were included in the study for comparative
111 purposes.

112

113 **Genomic characterisation of EIEC isolates**

114 *Whole genome sequencing*

115 Whole genome sequencing of the EIEC strains was carried out to reach the coverage of at
116 least 20X per isolate. The genomes of the EIEC strains EF432, CNM-2113/13 and 6.81
117 were sequenced with an Ion Torrent Personal Genome Machine (Life Technologies,
118 Carlsbad, USA). The genome of the EF432 was sequenced in six different runs (three runs
119 of a 200 bp library and three runs of a 400 bp library) while the genomes of CNM-2113/13
120 and 6.81 strains were sequenced in one and two 400 bp runs, respectively. Sequencing of
121 the EIEC strain H142690012 was carried out by the PHE Genome Sequencing Unit using
122 Nextera library preparation and the Illumina HiSeq 2500 in fast run mode according to
123 manufacturers' instructions. The sequencing reads have been uploaded in the EMBL-EBI
124 sequence database (EMBL European Nucleotide Archive accession no. ERP010762).

125 The reference sequences of EIEC 4608 (Acc. no. JTCO00000000), *Shigella sonnei* Ss046
126 (Acc. no. NC_007384, NC_007385, NC_009345, NC_009346, NC_009347), *S. boydii*
127 CDC3083-94 (Acc. no. CP001063, CP001058, CP001059, CP001060, CP001061,
128 CP001062), *S. dysenteriae* Sd197 (Acc. no. NC_007606, NC_007607, NC_009344) and
129 *S. flexnerii* 2a str. 301 (Acc. no. NC_004337, NC_004851) were retrieved from GenBank
130 database at NCBI.

131 *Bioinformatics analysis*

132 The sequencing reads of the EF432, CNM-2113/13, H142690012 and 6.81 isolates were
133 assembled in contigs by using SPADES *de novo* assembling tool version 3.5.0 [14] and
134 automatically annotated with PROKKA tool version 1.10 [15]. The two tools were operated
135 through a local instance of the bioinformatics framework Galaxy [16].

136 The contigs obtained through the assembly process from the EIEC strains and the
137 sequences of the EIEC reference strains retrieved from GenBank, were searched for the
138 serotype-associated genes using the blastn tool present on the Galaxy against a
139 precompiled database of reference sequences [17] provided by Dr. Flemming Scheutz at
140 the Statens Serum Institut, Copenhagen, DK.

141 The Multi Locus Sequence Typing was performed according to the scheme developed by
142 Wirth and colleagues [18] using the blastn tool to search the reference database of alleles
143 downloaded from the University of Warwick website [19]. The combinations of alleles of
144 the seven genes obtained through the blastn were translated into the corresponding
145 sequence types (ST) using the online tool located at the University of Warwick website.

146 The phylo-group assignment was performed *in silico* by using the blastn tool to query the
147 contigs of EIEC strains for the expected amplification products of the genes part of the
148 scheme developed by Clermont and colleagues [20]. The reference database of the
149 virulence genes of EIEC and *Shigella* strains has been compiled in house by merging the
150 gene sequences retrieved from the VFDB website [21] with those of the genes *virB*, *virF*,
151 *mxiE* and *ipgC* retrieved from GenBank (Gene ID: 1237991, 1238022, 876514 and
152 1238043, respectively), in a single multi fasta file and used with the blastn tool for the
153 screening of the genomes for the presence of *Shigella*/EIEC virulence genes.

154 The presence of the described pathoadaptative mutations was investigated by progressive
155 alignment of the annotated contigs of the three EIEC strains with the corresponding
156 regions on the *E. coli* K12 chromosome using the software MAUVE [22] and by means of

157 phenotypic assays (API). In particular, the presence and the integrity of the genes *cadA*,
158 *cadB*, *cadC*, *speA*, *speB*, *speC*, *speD*, *speE*, *speF*, *speG*, *nadA* and *nadB* (Gene IDs:
159 85676884, 85676885, 85676886, 1789307, 1789306, 87082193, 1786311, 1786312,
160 1786909, 85675033, 16128718, 16130499, respectively) was evaluated.

161 The identification of the presence of known replicons in the whole genome sequences was
162 achieved using the PlasmidFinder tool available on the CGE webserver [23] and used as
163 an indicator of the plasmid content of the isolates.

164

165 *Pulsed Field Gel Electrophoresis*

166 The pulsed field gel electrophoresis (PFGE) was performed with XbaI enzyme as
167 previously described [24]. The similarity evaluation of PFGE profiles was performed with
168 the Bionumerics software version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium) using
169 the algorithm UPGMA with tolerance and optimization set at 1.5%.

170

171 ***Phenotypic Characterization of EIEC in comparison with Shigella spp.***

172 *Growth curves*

173 Bacteria were grown statically O.N. in LB at 30°C, diluted 1:10 and evaluated
174 spectrophotometrically. Cultures were diluted to OD₆₀₀ 0.1 and plated on LB agar plates
175 in order to verify the equivalence between the readings and the number of viable cells in
176 the inocula. Each OD₆₀₀ 0.1 culture was diluted 1:100 in the same medium (LB) and five
177 0.1 ml aliquots were dispensed in 96-wells microplates and incubated at 37°C. Growth
178 was monitored by OD₆₀₀ using the Wallac Victor counter (Perkin Elmer, Waltham, US-
179 MA). The data obtained were used to calculate the G_t (generation time) and the lag delay.
180 To perform the auxotrophy test for nicotinic acid, the strains were grown in M9 minimal
181 medium supplemented with 10 µg/ml thiamine, 0.2% glucose and w/o 40 µg/ml nicotinic
182 acid.

183

184 *Motility assays*

185 Motility assays (swimming) were carried out using Tryptone swim plates (1% tryptone,
186 0.5% NaCl, 0.3% agar). In detail single colonies of the test strains grown overnight on LB
187 agar plates were inoculated at the surface by using a sterile needle and incubated for 16 h
188 at 37°C. The diameters of the swimming motility zones were measured and the results
189 recorded as mean values of three replicates.

190

191 *Biochemical characterization*

192 The EIEC strains were analysed for their biochemical profiles through the Analytical Profile
193 Index (API) 20E test (BioMérieux SA, France) according to the manufacturer's instructions.

194

195 **Results**

196 ***Genomic characterisation of EIEC isolates***

197 The draft genomes of the strains EF432, CNM-2113/13 and H142690012 and the
198 reference EIEC strain 6.81, together with that of the EIEC strain 4608 obtained from
199 GenBank (Acc. No. JTCO00000000) were analysed for the identification of the genes
200 associated with the *E. coli* O and H antigens to determine or confirm the serotypes. The
201 EIEC strains from the European outbreaks and the sporadic case showed the presence of
202 the genes associated with the O96:H19 serotype, while the genomes of the reference
203 strains 4608 and 6.81 revealed the presence of genes encoding the serotypes O143:H26
204 and O160:H26, respectively. It has to be noted that the latter strain was originally reported
205 as belonging to serogroup O115 [11]. The three EIEC strains O96:H19 all belonged to the
206 same sequence type ST-99, different from those of the reference EIEC strains 4608 and
207 6.91, respectively belonging to ST-280 and ST-279. Similarly, the three O96 strains were
208 of B1 phylo-group, while the reference EIEC strains were all of phylo-group E.

209

210 *Virulence genes asset of the EIEC strains*

211 The presence of the virulence genes so far described in EIEC and *Shigella* was
212 investigated in the EIEC O96:H19 strains and compared to the virulence genes asset of
213 the two reference EIEC strains 6.81 and 4608 and with the whole genomes of the strains
214 of *Shigella sonnei* Ss046, *S. boydii* CDC3083-94, *S. dysenteriae* Sd197, *S. flexneri* 2a str.
215 301. The complete results are reported in Table S1 in supplementary material.

216 The plasmid-borne virulence genes were present in all the EIEC strains analysed. These
217 included those encoding the type three secretion system (TTSS), *mxi* and *spa*, as well as
218 the *ipaA-H* and *ipgA-F* genes, encoding TTSS-secreted effectors, *icsA*, *icsB*, *icsP* and
219 *virA*, altogether responsible for the cytoskeleton reorganisation and mobility of the bacteria
220 inside the invaded host cells, *ospG*, whose product interferes with the innate immune
221 responses, and the regulatory genes *virF* and *virB*, responsible for activating the
222 transcription of all the other virulence genes (Table S1). All the plasmid genes were also
223 present in all the *Shigella* strains assayed with the exception of *senB*, encoding a toxin
224 involved in the early on-set of symptoms of gastrointestinal disease, which was absent
225 from the *S. flexneri* and *S. dysenteriae* strains assayed (Table S1).

226 The whole set of the chromosomal *gsp* genes (*gspC-M*), encoding a type two secretion
227 system typically present in *Shigella dysenteriae* and *S. boydii* was present in the genome
228 of all the strains assayed but in the chromosomes of *S. sonnei* Ss046 and *S. flexneri* 2a
229 str. 301 (Table S1). All the EIEC strains analysed were negative for the presence of the
230 *set1A* and *set1B* genes encoding the *Shigella* enterotoxin 1 and *pic* gene, which encodes
231 an autotransporter protease with mucinase and haemagglutinin activity involved in the
232 colonisation process. The latter three genes occupy the same chromosomal region in the
233 *S. flexnerii* 2a strain 301. The three EIEC O96:H19 strains, similarly to the EIEC strain
234 6.81, were negative for the presence of the *iucABC* and *iutA* genes encoding the

235 aerobactin system, playing an important role in the iron uptake especially during
236 intracellular growth, which was instead present in EIEC strain 4608. The *sigA* gene,
237 encoding a protease involved in intestinal fluid accumulation was absent in all the strains
238 tested, with the exception of the reference strains EIEC 4608 and *S. sonnei* Ss46 (Table
239 S1). Finally, the *gtr* genes associated with the lipopolysaccharide assembly could not be
240 found in the sequences of all the EIEC and *Shigella* strains tested, with the exclusion of
241 the *S. flexnerii* 2a strain 301 (Table S1).

242

243 *Analysis of the pathoadaptative mutations*

244 *Shigella* and EIEC are known to have accumulated mutations inactivating genes whose
245 product is thought to be detrimental to the survival of the pathogen in the cellular
246 environment. Such mutations have been hypothesized to favour the intracellular invasion
247 and persistence. These events are defined pathoadaptative mutations and the genes
248 involved, such as those of the *cadBA* operon, its *cadC* regulator and the *spe* genes
249 intervening in the polyamine metabolism are termed antivirulence genes [25]. The integrity
250 of the coding sequences of the mentioned genes has been evaluated in the EIEC strains
251 in this study. In contrast to what is normally reported for EIEC, the three O96:H19 strains
252 didn't show mutations in these loci. As expected, the presence of pathoadaptative
253 mutations in these genes were confirmed for the EIEC strains 4608 and 6.81 used as
254 references in this study. This result is in agreement with the positivity for the lysine
255 decarboxylase activity detected through the API biochemical tests of the EIEC O96:H19.
256 The latter strains were also negative for the presence of mutations in the *nadA*, *nadB* and
257 *argT* genes, commonly observed in *Shigella* and EIEC.

258

259 *Plasmid profiles*

260 The plasmid profiles of the EIEC strains were determined by analysing their whole genome
261 sequences with the PlasmidFinder tool available on the CGE webserver
262 (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>) [23]. The results are reported in Table 1.
263 All the isolates tested showed the presence of the FII replicon (Acc. No. AY458016)
264 suggesting its correspondence with the invasion plasmid. As a matter of fact, the same
265 replicon characterises the only EIEC virulence plasmid whose sequence is available in
266 GenBank (Acc. No. NC_010719) as well as those of *Shigella* [23]. All the EIEC O96:H19
267 shared the presence of two additional replicons, ColRNAI and FIB (Acc. No. DQ298019
268 and AP001918, respectively) indicating the presence of as many plasmids.
269 The CNM-2113/13 and 6.81 strains showed the presence of two different B/O/K/Z
270 replicons, described in plasmids harbouring beta-lactamases coding genes [23]. A replicon
271 Q1 was identified in the sequence of the H142690012 strain only. Replicons
272 corresponding to two different small plasmids could be identified in the genomes of CNM-
273 2113/13, H142690012 and 6.81 strains.

274

275 *Genomic correlation among the EIEC strains*

276 Pulsed field gel electrophoresis (PFGE) was performed to estimate the correlation
277 between the genomes of the EIEC strains included in the study. The PFGE profiles of
278 three EIEC isolates obtained during the outbreak occurred in Italy in 2012 (EF432, EF433
279 and EF434), the EIEC strain isolated Spain in 2013 (CNM-2113/13) and the strain
280 responsible for the outbreak occurred in UK in 2014 (H142690012) were produced and
281 compared with those of the two reference EIEC strains 6.81 and 4608. The results are
282 shown in Figure 1. As expected, the PFGE profiles of the three Italian isolates clustered
283 with 100% identity and showed a high similarity level with the strains CNM-2113/13 and
284 H142690012 strains, while being only distantly related to the EIEC reference strains 6.81
285 and 4608.

286

287 ***Phenotypic Characterization of EIEC in comparison with Shigella spp.***

288 Many EIEC share phenotypic features with strains of *Shigella*. Characteristically, they do
289 not ferment lactose [7] and lack lysine decarboxylase activity [25]. Additionally, in some
290 cases EIEC belong to O groups identical to the typical *Shigella* O-antigens [7].

291

292 *Fitness*

293 EIEC are considered an intermediate step in the transition of *E. coli* towards a full-blown
294 *Shigella* phenotype. This evolutionary process is characterised by an extensive and
295 progressive genetic decay through gene deletion and accumulation of pseudogenes. Such
296 a process involves, among the others, determinants coding for carbon sources utilization,
297 such as transporters or membrane proteins [26]. Therefore, the bacterial growth curve
298 analysis provides information about the magnitude of gene decay since the latter reduces
299 the bacterial metabolic activity.

300 Based on these considerations, we compared the growth curves of EF432, CNM-2113/13
301 and H142690012 strains, cultured in LB medium, at 37°C in microplate, with those of the
302 *E. coli* K12 laboratory strain MG1655, the EIEC strains 4608 and 6.81, the *S. flexneri*
303 strain M90T and the *E. coli* natural isolate ECOR1 used as reference isolates. In particular,
304 we analysed the Generation time (Gt), the stationary and the lag phase. The growth
305 analysis showed no significant differences in the Gt of the three EIEC investigated and the
306 EIEC 6.81 and ECOR1 strains used for comparison (from 23.8 to 23.9 minutes). The EIEC
307 strain 4608 and *E. coli* K12 MG1655 showed similar Gt (25.2 and 24.8 minutes) while the
308 highest value characterised the growth of the *Shigella* strain M90T (26.2 minutes) (Table
309 S2). Furthermore, the A_{600} of each strain after prolonged incubation (16 h), i.e. stationary
310 phase, were also considered and no significant differences were found. Finally, the lag
311 phase analysis evidenced that the ECOR1 natural isolate displayed the promptest

312 adaptation to fresh medium followed by the three EIEC subject of this study (4 minute
313 delay) (Figure 2) and the EIEC reference strains 4608 and 6.81 (13 minute delay). Finally,
314 the *E. coli* K12 MG1655 and *S. flexneri* M90T strains showed the longest lag phase before
315 the cultures entered the exponential growth (20 and 43 minutes, respectively). These
316 results suggest that strains EF432, CNM-2113/13 and H142690012 have a high metabolic
317 versatility and could be more efficient at exploiting the available nutritional elements in the
318 medium than the *E. coli* K12 strain and the reference EIEC and *S. flexneri* strains. This
319 consideration was further confirmed by the analysis of the *nic* phenotype, assessed by the
320 ability to grow in the absence of nicotinic acid and determined by the presence of
321 functional *nad* genes [27]. The *nic* phenotype is considered a final step characteristic in
322 the evolutionary route of EIEC towards *Shigella*, since *Shigella* typically lacks a de novo
323 pathway for the synthesis of nicotinamide adenine dinucleotide and requires nicotinic acid
324 for growth in minimal medium while only some EIEC strains have this requirement [27].
325 Strains EF432, CNM-2113/13 and H142690012 grow on minimal medium without nicotinic
326 acid indicating that *nad* genes are functional and correctly expressed in all the three EIEC
327 strains analysed.

328

329 *Biochemical characterisation*

330 The three EIEC strain assayed EF432, CNM-2113/13 and H142690012 shared the same
331 biochemical profile when tested through the Analytical Profile Index (API) 20E test. In
332 particular, they were positive for the β -galactosidase and lysine decarboxylase (LDH)
333 activity and were able to ferment glucose, mannose, sorbitol, rhamnose, sucrose,
334 melibiose and arabinose. The observed phenotypes resulted in the API code 5004572,
335 identical for all the three strains, which corresponded to a good identification of the
336 *Escherichia coli* species (id=90.7%).

337

338 *Motility test*

339 The lack of motility constitutes important taxonomic and diagnostic criteria used to
340 differentiate *Shigella* from other members of the *Enterobacteriaceae*, being the former
341 non-motile, similarly to the majority of EIEC strains. In order to analyse the motility
342 phenotype of the EIEC O96:H19, we performed a swim assay using the EF432, CNM-
343 2113/13 and H142690012 strains and included the MG1655, 4608, 6.81, M90T and
344 ECOR1 as reference strains. We inoculated the swim plates and, after 16 hours of
345 incubation, no swimming was observed for M90T, 4608, 6.81 and H142690012, while
346 MG1655 showed the strongest ability to swim (33 mm diameter) (Figure 3). ECOR1
347 showed an intermediate level of mobility (15 mm diameter) while EF432 and CNM-2113/13
348 showed residual mobility (5.5 and 4.1 mm) indicating a partial but significant impairment of
349 flagella biosynthesis and functionality.

350

351 **Discussion**

352 EIEC infections share with those caused by bacteria belonging to genus *Shigella* the
353 pathophysiological features and modes of transmission [7]. While the burden of EIEC
354 infections is not known mainly due to mis-diagnosis, most of what we know derives from
355 studies done on the prevalence of *Shigella* infections among the populations living in
356 underdeveloped countries, where they cause millions of human cases of disease with
357 more than one million deaths, yearly [4].

358 In addition to the heavy toll paid by low-income countries, *Shigella* infection is the third
359 most common cause of bacterial gastroenteritis in the United States, after *Salmonella* and
360 *Campylobacter* infections and followed by *E. coli* O157, *Vibrio* and *Yersinia* [28, 29].

361 No immunity to shigellosis has been described for specific groups, but certain individuals
362 are at increased risk of getting infected. Children below five acquire *Shigella* infection at

363 the highest rate [30] and other immunocompromised groups, such as persons infected
364 with HIV, suffer from *Shigella* infections much more commonly than other individuals [31].
365 Insights into the phylogeny of EIEC and *Shigella* have been obtained from studies based
366 on the analysis of the variations accumulated in the DNA sequence of housekeeping
367 genes [18, 32]. Such studies provide evidences that these pathogens should be
368 considered as two different steps of the same evolutionary pathway rather than two
369 separated bacterial species [2, 18, 32]. It is likely that EIEC are precursors of *Shigella*,
370 which could have emerged following acquisition of the plasmid containing the invasion
371 genes [32], by a commensal *E. coli*, eventually transforming into the strains known as
372 *Shigellae* following the accumulation of pathoadaptative mutations that facilitated the
373 intracellular lifestyle [25]. In particular, according to the evolutionary relationships between
374 the two species, the strains of *Shigella* seem to be derived from *E. coli* belonging to the
375 ST-280 clonal complex (Cplx) [18].

376 In industrialised countries the information on EIEC disease are scanty. These infections
377 are believed to be mostly imported from developing countries and with limited possibility to
378 spread due to their inter-human transmission cycle [6]. Recently, a study was published
379 analysing the frequency of enteric pathogens in stool samples of patients affected with
380 acute gastroenteritis throughout Europe, reporting a very low prevalence for EIEC or
381 *Shigella* (10 positive out of 709 samples analysed) [33]. Nevertheless, recently an upsurge
382 in cases of EIEC infections in the European Union has been observed [8, 9]. Two large
383 outbreaks have been reported, in years 2012 and 2014, in Italy [8] and the UK [9],
384 respectively, both involving a high number of cases. It is noteworthy that the two episodes
385 have been linked to the consumption of contaminated food [8, 9]. In addition to these two
386 outbreaks, one sporadic case has been registered in Spain in 2013. We have
387 characterised a set of three EIEC strains isolated from the two outbreaks and the sporadic
388 case in Spain and found that all of them belonged to serotype O96:H19, a rare serotype

389 first attributed to the EIEC strain that caused the Italian outbreak in 2012 [8]. Additionally,
390 we have found that the three EIEC all belonged to the ST-99 that in turn does not belong
391 to any known ST Cplx and it is only distantly related to the ST-280 Cplx, having only one of
392 the alleles of the scheme in common.

393 These observations, together with the observed difference in the phylo-group of EIEC
394 O96, when compared to that of the EIEC reference strains, suggested that the EIEC
395 O96:H19 could be the result of a recent event of acquisition of the invasion plasmid. This
396 was confirmed by the analysis of the virulence genes content and pathoadaptative
397 mutation pattern. The whole genomes of the three isolates was determined and compared
398 with a database containing all the known virulence gene of *Shigella*/EIEC. The three EIEC
399 O96:H19 showed the expected pattern of virulence genes displaying the complete
400 plasmid-borne virulome of the EIEC and *Shigella* strains used as controls (Table S1).
401 Nevertheless, the genome analysis showed that they had not acquired any of the
402 described pathoadaptative mutations.

403 In order to support the hypothesis that the three EIEC O96:H19 were closer to *E. coli* than
404 to *Shigella*, we have characterised them phenotypically with respect to their ability to move
405 and diffuse into agar layers. As expected, some residual movement was observed in two
406 out of the three EIEC O96:H19 in comparison to all the EIEC and *Shigella* control strains
407 that were non-motile. Also, the observed prototrophic behaviour of the EIEC O96:H19
408 towards the use of nicotinic acid for growth seems to confirm this hypothesis. The
409 auxotrophism for this compound is an evolutionary feature in *Shigella*, and is partially
410 present in the EIEC strains described so far [27]. Even more strikingly, the three isolates
411 showed a more rapid entry into the log phase than the *Shigella* and EIEC controls, but
412 very similar to how observed with the *E. coli* natural isolate ECOR1 strain included in the
413 experiments (Figure 2). This latter feature is interesting and may, at least partially, explain
414 the described association of the two outbreaks with a food vehicle. The Italian episode

415 was epidemiologically linked to the consumption of cooked vegetables [8], while the
416 outbreak occurred in the UK was reported as being caused by the consumption of salad
417 [9]. In both cases the superior ability of the EIEC O96:H19 to grow with an “*E. coli* style”
418 may have been driving the overgrowth in the food vehicle and could explain the size of the
419 two episodes. In fact none of the known EIEC or *Shigella* would have had the possibility to
420 reach a bacterial load able to cause such a high number of people affected.

421 In conclusion, our findings support the hypothesis that the EIEC O96:H19 that caused
422 sporadic cases and outbreaks of infections in the EU belong to an emergent clone never
423 described previously. The genomic characterisation of these isolates, carried out by
424 PFGE, further confirms this hypothesis (Figure 1). Additionally, our results seem to
425 indicate that EIEC may emerge as the result of the continuous acquisition, by commensal
426 *E. coli*, of the plasmid containing the invasion determinants and that, under certain
427 circumstances, as in the case of the *E. coli* strains of ST-280 Cplx, this process
428 progresses with the accumulation of pathoadaptative mutations and the loss of some of
429 the *E. coli* characteristics, such as the rapid entry into the exponential growth phase, more
430 adapted to an out-of-the-cell lifestyle, eventually resulting in the emergence of *Shigellae*
431 (Figure 4).

432 The hypothesis of a stepwise model for the emergence of *Shigella* from *E. coli* and the
433 possibility that the process may reiterate from time to time could explain the emergence
434 and the sudden appearance of the highly virulent EIEC O96:H19 clone and paves the way
435 to the possible emergence of new EIEC clones causing outbreaks of infections in the
436 future.

437

438 **References**

439

440 1 Nataro JP, Kaper JB. Diarrheagenic escherichia coli. *Clinical microbiology reviews*. 1998;
441 **11**: 142-201.

- 442 2 van den Beld MJ, Reubsaet FA. Differentiation between shigella, enteroinvasive
443 escherichia coli (eiec) and noninvasive escherichia coli. *European journal of clinical*
444 *microbiology & infectious diseases : official publication of the European Society of Clinical*
445 *Microbiology*. 2012; **31**: 899-904.
- 446 3 Sansonetti PJ. Rupture, invasion and inflammatory destruction of the intestinal barrier
447 by shigella, making sense of prokaryote-eukaryote cross-talks. *FEMS microbiology*
448 *reviews*. 2001; **25**: 3-14.
- 449 4 Kotloff KL, Winickoff JP, Ivanoff B, et al. Global burden of shigella infections:
450 Implications for vaccine development and implementation of control strategies.
451 *Bulletin of the World Health Organization*. 1999; **77**: 651-666.
- 452 5 Vieira N, Bates SJ, Solberg OD, et al. High prevalence of enteroinvasive escherichia coli
453 isolated in a remote region of northern coastal ecuador. *The American journal of*
454 *tropical medicine and hygiene*. 2007; **76**: 528-533.
- 455 6 Niyogi SK. Shigellosis. *Journal of microbiology*. 2005; **43**: 133-143.
- 456 7 Tozzoli R, Scheutz, F. Diarrheagenic escherichia coli infections in humans. In: Stefano
457 M, ed. *Pathogenic escherichia coli: Molecular and cellular microbiology*: Caister
458 Academic Press 2014.
- 459 8 Escher M, Scavia G, Morabito S, et al. A severe foodborne outbreak of diarrhoea linked
460 to a canteen in italy caused by enteroinvasive escherichia coli, an uncommon agent.
461 *Epidemiology and infection*. 2014; **142**: 2559-2566.
- 462 9 PHE PHE. Foodborne illness in humans: General outbreaks in england and wales in
463 2014. London, UK: Public Health England 2014.
- 464 10 Luscher D, Altwegg M. Detection of shigellae, enteroinvasive and enterotoxigenic
465 escherichia coli using the polymerase chain reaction (pcr) in patients returning from
466 tropical countries. *Molecular and cellular probes*. 1994; **8**: 285-290.
- 467 11 Sansonetti PJ, d'Hauteville H, Formal SB, Toucas M. Plasmid-mediated invasiveness of
468 "shigella-like" escherichia coli. *Annales de microbiologie*. 1982; **133**: 351-355.
- 469 12 Guyer MS, Reed RR, Steitz JA, Low KB. Identification of a sex-factor-affinity site in e.
470 Coli as gamma delta. *Cold Spring Harbor symposia on quantitative biology*. 1981; **45 Pt**
471 **1**: 135-140.
- 472 13 Ochman H, Selander RK. Standard reference strains of escherichia coli from natural
473 populations. *Journal of bacteriology*. 1984; **157**: 690-693.
- 474 14 Bankevich A, Nurk S, Antipov D, et al. Spades: A new genome assembly algorithm and
475 its applications to single-cell sequencing. *Journal of computational biology : a journal of*
476 *computational molecular cell biology*. 2012; **19**: 455-477.
- 477 15 Seemann T. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics*. 2014; **30**:
478 2068-2069.
- 479 16 Goecks J, Nekrutenko A, Taylor J, Galaxy T. Galaxy: A comprehensive approach for
480 supporting accessible, reproducible, and transparent computational research in the life
481 sciences. *Genome biology*. 2010; **11**: R86.
- 482 17 Joensen KG, Tetzschner AM, Iguchi A, Aarestrup FM, Scheutz F. Rapid and easy in silico
483 serotyping of escherichia coli using whole genome sequencing (wgs) data. *Journal of*
484 *clinical microbiology*. 2015.
- 485 18 Wirth T, Falush D, Lan R, et al. Sex and virulence in escherichia coli: An evolutionary
486 perspective. *Mol Microbiol*. 2006; **60**: 1136-1151.
- 487 19 Achtman M, Velayudhan V., Zhou Z. *Mlst databases at university of warwick*.
488 <http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>
- 489 20 Clermont O, Christenson JK, Denamur E, Gordon DM. The clermont escherichia coli
490 phylo-typing method revisited: Improvement of specificity and detection of new phylo-
491 groups. *Environmental microbiology reports*. 2013; **5**: 58-65.

- 492 21 MOH Key Laboratory of Systems Biology of Pathogens IoPB, CAMS&PUMC, Beijing,
493 China. <http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Shigella>
- 494 22 Rissman AI, Mau B, Biehl BS, Darling AE, Glasner JD, Perna NT. Reordering contigs of
495 draft genomes using the mauve aligner. *Bioinformatics*. 2009; **25**: 2071-2073.
- 496 23 Carattoli A, Zankari E, Garcia-Fernandez A, et al. In silico detection and typing of
497 plasmids using plasmidfinder and plasmid multilocus sequence typing. *Antimicrobial*
498 *agents and chemotherapy*. 2014; **58**: 3895-3903.
- 499 24 EFSA. Molecular typing of verocytotoxin-producing e. Coli (vtec) strains isolated from
500 food, feed and animals: State of play and standard operating procedures for pulsed
501 field gel electrophoresis (pfge)typing, profiles interpretation and curation.
502 *www.efsa.europa.eu/publications*. 2014; **European Food Safety Authority supporting**
503 **publication 2014:EN-704**.
- 504 25 Prosseda G, Di Martino ML, Campilongo R, et al. Shedding of genes that interfere with
505 the pathogenic lifestyle: The shigella model. *Research in microbiology*. 2012; **163**: 399-
506 406.
- 507 26 Feng Y, Chen Z, Liu SL. Gene decay in shigella as an incipient stage of host-adaptation.
508 *PloS one*. 2011; **6**: e27754.
- 509 27 Di Martino ML, Fioravanti R, Barbabella G, Prosseda G, Colonna B, Casalino M.
510 Molecular evolution of the nicotinic acid requirement within the shigella/eiec
511 pathotype. *Int J Med Microbiol*. 2013; **303**: 651-661.
- 512 28 Gupta A, Polyak CS, Bishop RD, Sobel J, Mintz ED. Laboratory-confirmed shigellosis in
513 the united states, 1989-2002: Epidemiologic trends and patterns. *Clin Infect Dis*. 2004;
514 **38**: 1372-1377.
- 515 29 Iwamoto M, Huang JY, Cronquist AB, et al. Bacterial enteric infections detected by
516 culture-independent diagnostic tests--foodnet, united states, 2012-2014. *MMWR*
517 *Morbidity and mortality weekly report*. 2015; **64**: 252-257.
- 518 30 Mohle-Boetani JC, Stapleton M, Finger R, et al. Communitywide shigellosis: Control of
519 an outbreak and risk factors in child day-care centers. *Am J Public Health*. 1995; **85**:
520 812-816.
- 521 31 Baer JT, Vugia DJ, Reingold AL, Aragon T, Angulo FJ, Bradford WZ. Hiv infection as a
522 risk factor for shigellosis. *Emerg Infect Dis*. 1999; **5**: 820-823.
- 523 32 Lan R, Alles MC, Donohoe K, Martinez MB, Reeves PR. Molecular evolutionary
524 relationships of enteroinvasive escherichia coli and shigella spp. *Infect Immun*. 2004;
525 **72**: 5080-5088.
- 526 33 Spina A, Kerr KG, Cormican M, et al. Spectrum of enteropathogens detected by the
527 filmarray gi panel in a multicentre study of community-acquired gastroenteritis.
528 *Clinical microbiology and infection : the official publication of the European Society of*
529 *Clinical Microbiology and Infectious Diseases*. 2015; **21**: 719-728.
- 530