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4   **Characterization of *Campylobacter jejuni* and *Campylobacter coli* broiler isolates**

5   **by whole genome sequencing**

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26   **Running title:** *C. jejuni* and *C. coli* characterization by WGS.

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28

29   **Abstract**

30   *Campylobacter* has been the most commonly reported cause of bacterial diarrhoeal

31   disease in humans in the EU since 2005 (EFSA, 2016). Most broiler batches at slaughter

32   are colonized with *Campylobacter* and the major source of infection is contaminated

33   poultry meat. The aim of this study was to characterize a selection of *C. jejuni* and *C.*

34   *coli* isolates from broilers through whole genome sequencing (WGS). A total of 16

35   isolates (*C. jejuni* = 12 and *C. coli* = 4) from five broiler farms from Catalonia

36   (northeastern Spain) were analyzed. A phylogenetic analysis based on 8420 SNPs

37   showed two main clusters grouping strains by species. Phenotypic resistances to

38   quinolones (100%), tetracycline (81%), streptomycin (75%), erythromycin (56%) and

39   gentamicin (13%) were found. All the isolates carried the C257T point mutation in the

40   subunit A of the DNA gyrase gene (Thr86Ile) conferring resistance to quinolones,

41   whilst all the isolates showing resistance to tetracycline carried the *tet(O)* gene. The

42   genes *aph(3′)-III* and *aadE* conferring resistance to aminoglycosides were identified in

43   the two isolates (one *C. jejuni* and one *C. coli*) resistant to streptomycin and gentamicin.

44   The point mutation A2075G on the 23S rDNA conferring high resistance to macrolides

45   was detected in three *C. coli* isolates. The CmeABC multidrug efflux pump was also

46   detected, both in *C. jejuni* and *C.coli* isolates. All *C. jejuni* and *C. coli* isolates were

47   positive for most of the 34 virulence-associated genes studied related to motility,

48   chemotaxis, adhesion and invasion. Interestingly, the *wlaN* gene involved in the

49   Guillain-Barré syndrome, was found in two isolates. The results underline the power of

50   WGS for investigation of virulence, clonality and antimicrobial resistance in

51   *Campylobacter*.

52

53

Introduction

Since 2005 *Campylobacter* has outnumbered *Salmonella* as the most commonly reported cause of bacterial diarrhoeal disease in humans in the EU (EFSA 2014). *C. jejuni* and *C. coli* are responsible for the vast majority of infections (Eberle & Kiess 2012), which may subsequently lead to serious neuropathy such as Guillain-Barré syndrome (Crushell et al. 2004). The majority of *Campylobacter* infections in humans are sporadic and self-limiting which complicates the determination of the true incidence rate (Hänninen et al. 2000). Due to the self-limiting behavior of the disease antimicrobial treatment is only indicated in severe cases where fluoroquinolones and macrolides are the drugs of choice (Butzler 2004; Moore et al. 2006).

In the majority of the EU countries most of the broiler batches are colonized with *Campylobacter* at slaughter and the main source of campylobacteriosis in humans is chicken meat, which can account for up to 70% of cases (Boysen et al. 2014). The prevention of broiler flock colonization has therefore become a food safety priority in the EU (EFSA, 2011), that is reflected by the new regulation (amendment of Annex I to EC regulation No 2073/2005 as regards *Campylobacter* in broiler carcasses) that may enter into force in 2018.

The pathogenicity of *Campylobacter* strains have been linked to multiple factors including host susceptibility and, more importantly, the expression of different virulence factors and resistance to antimicrobials. Several putative virulence factors have been identified in *Campylobacter* species that contribute to motility, intestinal adhesion, colonization, toxin production and tissue invasion (Dasti et al. 2010; Bolton 2015). Also, multidrug-resistant *C. jejuni* and *C. coli* have been reported worldwide

79   from farm animals and retail meats, including poultry and swine (Zhao et al. 2010;

80   Datta et al. 2003).

81

82   Phenotypic methods have been widely used to characterize *C. jejuni* and *C. coli* strains.

83   However, these methods have mostly been replaced by genotypic methods that are more

84   accurate and have higher discrimination power, such as pulsed-field gel electrophoresis

85   (PFGE) and multilocus sequence typing (MLST) (Pfaller 1999). Nevertheless, with the

86   advent of next-generation sequencing, the possibility of generating high-resolution full

87   genome data is being increasingly used to differentially characterize strains. This

88   technology allows for a rapid identification of a broad range of genotypic traits of the

89   isolates, such as their pool of virulence and antimicrobial resistance determinants. It has

90   proven useful in gaining insight into the epidemiology of *Campylobacter* and predicting

91   its antimicrobial resistance (Llarena et al 2017; Zhao et al 2015). Hence, the aim of this

92   study was to take advantage of whole genome sequencing (WGS) to in-depth

93   characterize a subset of *C. jejuni* and *C. coli* isolates from broilers obtained from a

94   longitudinal study involving different farms. The characterization included the

95   determination of the MLST genotype, the identification of virulence and antimicrobial

96   determinants as well as a phylogenetic study of the isolates through the discovery of

97   single nucleotide polymorphisms (SNPs) between the different strains analyzed.

98

99   **Materials and Methods**

100   ***Isolates***

101   A total of 16 poultry isolates (*C. jejuni* =12 and *C. coli*= 4) from five broiler farms

102   were included in the study. The isolates were selected from *Campylobacter* positive

103   flocks of a broad two-year longitudinal study (2011-2013), where six to seven flocks

4

104   were studied each year by cloacal swab sampling a subset of birds. Selection of the

105   isolates was performed according to their PFGE patterns (Supplementary Fig. 1) and an

106   antimicrobial multidrug-resistant profile by disc diffusion (unpublished data). The five

107   different farms (A, B, C, D and E) were located in Catalonia (northeastern Spain).

108   Poultry houses had a capacity of 12,000 to 46,000 birds, and age of sampled birds

109   ranged 18 to 39 days. *Campylobacter* isolation and identification was performed as

110   previously described (Urdaneta et al., 2015). Isolates were preserved in brain heart

111   infusion broth (BHI, Merck KGaA, Darmstadt, Germany), with 20% glycerol at -80ºC

112   until used and fresh cultures of the isolates were prepared on Columbia blood agar

113   plates (bioMérieux, Marcy-l'Etoile, France). Plates were incubated at 37 °C for 48 h

114   under microaerobic conditions using a microaerobic atmosphere generator (Anaerocult®

115   C, Merck, Darmstadt, Germany).

116

117   ***Antimicrobial susceptibility testing***

118   Isolates were tested for antimicrobial susceptibility using a minimum inhibitory

119   concentration (MIC) based broth microdilution (VetMIC GN-mo; National Veterinary

120   Institute, Uppsala, Sweden) for the following antimicrobial agents: nalidixic acid (1 to

121   64 mg/L), ciprofloxacin (0.06 to 8 mg/L), tetracycline (0.12 to 16 mg/L), streptomycin

122   (0.5 to 64 mg/L), gentamicin (0.12 to 16 mg/L), and erythromycin (0.5 to 64 mg/L). *C.*

123   *jejuni* ATCC 33560 and *C. coli* ATCC 33559 were used as control strains. An isolate

124   was considered multidrug-resistant when showing resistance to three or more non-

125   related antimicrobials. Isolates were considered to be susceptible or resistant based on

126   epidemiological cutoff values according to EUCAST guidelines (www.eucast.org).

127   When reporting data using EUCAST epidemiological cut-off values, bacteria should be

128 reported as 'wild-type' (WT) or 'non-wild-type' (non-WT) (Schwarz et al. 2010). For

129 simplicity of the terms, susceptible and resistant has been used here.

130

131 ***Whole genome sequencing (WGS) and assembly***

132 Genomic DNA was extracted using QIAamp DNA mini kit (QIAGEN) according to the

133 manufacturer´s instructions. The libraries were prepared with Nextera XT DNA sample

134 preparation kit (Illumina Inc., San Diego, CA, cat. no. FC-131-1024) followed by

135 multiplexed paired-end sequencing with a read length of 2 × 251 bp, using Illumina's

136 MiSeq platform (Illumina).

137

138 The raw reads were trimmed and cleaned for adapters, and assembling was performed

139 using the online tool Assembler v1.2 with default parameters. All these steps are

140 integrated in a pipeline available at the Center for Genomic Epidemiology (CGE)

141 (www.genomicepidemiology.org). Contiguous assemblies were analyzed using the

142 CLCbio's Genomics Workbench v6.5 (CLCbio's, Aarhus, Denmark).

143

144 The raw sequence dataset is available in the NCBI database with Bioproject Accession

145 number PRJNA385807.

146

147 ***Analysis of resistance and virulence-associated genes***

148 ResFinder v2.1 (https://cge.cbs.dtu.dk/services/ResFinder/) and MyDbFinder v1.1

149 (https://cge.cbs.dtu.dk/services/MyDbFinder/), both available at the CGE were used for

150 identification of resistance and virulence genes, respectively. All strains were subjected

151 to analysis of the presence of resistance determinants to quinolones, tetracyclines,

152 aminoglycosides and β-lactams. *C. jejuni* and *C. coli* strains were tested for 34

153   virulence-associated genes; the identifiers of each of the genes analyzed and the

154   homology analyses are detailed in Supplementary Tables 2 and 3, respectively. The

155   presence of several virulence-associated genes related to motility (eight), chemotaxis

156   (five), adhesion (four), invasion (three), cytolethal distending toxin (three), multidrug

157   and bile resistance (three), stress response and survival (two), iron uptake (two), capsule

158   (two), Guillian-Barré syndrome (one) and hippuricase (one), was assessed (Koolman et

159   al. 2015). All genes were identified with a selected identity threshold of 80% (Zankari

160   et al. 2012), and a minimum coverage of 20% of the query sequence length. The

161   presence of the *flaA* and *flaB* motility genes was confirmed by PCR with specific

162   primers (Koolman et al. 2015).

163

164   In order to analyze the presence/absence of specific mutations related to antibiotic

165   resistance, the raw fastq files for each of the isolates were aligned with bwa mem

166   algorithm (Li & Durbin 2009) with the corresponding reference genome (AL111168 for

167   *C. jejuni* and CP011015 for *C. coli*). The alignment files and the corresponding

168   annotated reference genome were inspected manually using Tablet as the visualizing

169   tool (Milne et al. 2013). Only mutations that appeared with frequency higher than 0.5%

170   were considered.

171

172   **Identification of SNPs**

173   The SNP discovery was done using the CSI Phylogeny v1.4 pipeline CGE (Kaas et al.

174   2014). Briefly, the paired-end reads from each of the isolates were reference-aligned

175   using strain 12 for *C. jejuni* and 4 for *C. coli* with BWA v.0.7.2 software (Li & Durbin

176   2009). SNP calling was done with SAMtools v.0.1.18 ('mpileup' method, Li & Durbin

177   2009) and filtering was done with BEDTools (Quinlan & Hall 2010). To select the valid

178 SNPs, the same criteria previously described was followed (Kaas et al. 2014). SNPs

179 were filtered out if the mapping quality was below 25 or the SNP quality was below 30,

180 and if they were called within the vicinity of 10 bp of another SNP (pruning). To

181 perform the phylogenetic analysis, concatenation of the sequences and subsequent

182 aligning with MUSCLE was done (Edgar 2004). Maximum likelihood trees were

183 created   using   CSI   Phylogeny   1.4   (available   at

184 https://cge.cbs.dtu.dk/services/CSIPhylogeny/) (Price et al. 2010) using the following

185 parameters: minimum depth at SNP position was set at 10x, the minimum SNP quality

186 accepted was 30 and the minimum read mapping quality allowed was 25. The reference

187 genome used was *C. coli* (Accession number CP011015).

188

189 ***Multilocus sequence typing (MLST)***

190 The *de novo* assembled contigs were used to identify the multilocus sequence types

191 (ST) and clonal complexes (CC) using the web server MLST v1.8 available at the CGE

192 website (www.genomicepidemiology.org).

193

194 **Results**

195 ***Whole genome sequencing***

196 The isolates were sequenced to an average coverage that varied from 49,73 to 127,2 x

197 (Supplementary Table 1). From the assembled contigs of the sixteen isolates, nine

198 previously-described sequence types (ST) were recognized (Fig. 1). Besides, two novel

199 STs   not   previously   reported   in   the   PubMLST   database

200 (http://pubmlst.org/campylobacter/) were identified, ST8586 (E13, *C. jejuni* isolate) and

201 ST8578 (E16, *C. coli* isolate). All the STs found belong to five different CC. The most

202 frequent CC in *C. jejuni* were CC257 and CC21 followed by CC206, whilst the less

203 prevalent was CC45 with only one *C. jejuni* isolate from ST45 belonging to this group.

204 All of the *C. coli* isolates belonged to the same clonal complex (CC828), which

205 included three different STs (ST827, ST854 and ST8578).

206

207 Comparison of the sequences obtained from the sixteen isolates allowed the

208 identification of a total of 8420 SNPs, which were used to perform the phylogenetic

209 analysis. The representation of the SNP tree is depicted in Fig. 1 and a heatmap

210 representing the SNP counts between the genomes is shown in Supplemental Fig. 2.

211 None of the isolates analyzed were identical. The isolates showed distribution in two

212 main clusters, with all strains grouped by species.

213

214 ***Antimicrobial susceptibility***

215 The phenotypic antimicrobial susceptibility patterns determined for the sixteen strains

216 analysed is shown in Table 1. All the strains showed a multidrug resistant profile, with

217 resistance to quinolones (nalidixic acid and ciprofloxacin) being common to all of them.

218 The resistance to tetracycline was the second most commonly observed (81%) followed

219 by streptomycin and erythromycin resistance (75% and 56%, respectively). Among the

220 latter, one third of *C. jejuni* isolates were resistant, whilst all *C. coli* isolates showed

221 resistance to this antimicrobial. Gentamicin resistance was the less prevalent, detected

222 only in two isolates from farms A and B (13%).

223

224 In order to study the potential mechanisms of antimicrobial resistance, the assembled

225 genomes of each of the strains under study were investigated for particular patterns

226 known to be associated to resistance. Phenotypic antimicrobial resistances obtained with

227 the MIC analysis corresponded well for most of the isolates with the identification of

228   specific antimicrobial resistance genes detected by WGS (Table 1). All the isolates

229   carried the C257T point mutation in the subunit A of the DNA gyrase gene (Thr86Ile)

230   conferring resistance to quinolones. Other less common mutations in *gyrA* (Asp-90-Asn

231   and Ala-70-Thr) were not detected in any of the isolates. All the isolates showing

232   resistance to tetracycline carried the *tet(O)* gene. Within the two isolates resistant to

233   streptomycin and gentamicin, the genes *aph(3′)-III* and *aadE* conferring resistance to

234   aminoglycosides were identified. Three *C. coli* isolates were found to show a mutation

235   in the A2075G position of the 23S rDNA region, which confers a high level of

236   resistance to macrolides. The CmeABC multidrug efflux pump has been described as

237   the major efflux pump mechanism conferring resistance to a wide range of

238   antimicrobials, and it was identified in 15 out of the 16 isolates analyzed.

239

240   ***Virulence determinants***

241   All isolates of *C. jejuni* and *C. coli* were positive for almost all of the 34 virulence-

242   associated genes studied, including motility, chemotaxis, adhesion and invasion genes,

243   with few exceptions (Table 2). The flagellin genes were unexpectedly found absent in

244   most of the strains through WGS analysis. However, due to the known difficulty

245   associated to accurately assemble duplicated genes, the presence of these *flaA* and *flaB*

246   genes was assessed by PCR. Table 2 shows the experimentally-validated presence of

247   *flaB* in all the isolates and absence of *flaA* in one third of the isolates by specific PCR.

248   Only one isolate was negative for *cmeB* (component of the CmeABC efflux pump) and

249   another one for *cfrA* (gene involved in iron uptake), both were *C. jejuni* strains from

250   farm E. Remarkably, the *wlaN* gene, involved in the Guillain-Barré syndrome, was

251   detected in two *C. jejuni* isolates from different farms. As expected, the *hipO* gene was

252   not detected in any *C. coli* isolate.

253

255 **Discussion**

256 The whole-genome sequence data revealed that the *C. jejuni* and *C. coli* isolates

257 belonged to five different CC, all of them associated both with poultry and human

258 campylobacteriosis in many countries (*Campylobacter* PubMLST;

259 http://PubMLST.org). The two positive isolates to *wlaN* gene, related to the Guillain

260 Barré Syndrome, belonged to ST21 and to the novel ST8586, both from CC21. In the

261 *Campylobacter* PubMLST database, there are only seven isolates within CC21

262 associated with the Guillain-Barré syndrome and none of them belong to the widespread

263 ST21.

264

265 The comparison of the assembled genomes revealed a large number of nucleotide

266 changes (SNPs), among the isolates and the reference genomes. The identified

267 variations were used to study the phylogeny to infer the relationship among the isolates,

268 which were in concordance to the species they belong (Fig. 1). Not surprisingly, isolates

269 of identical ST were more closely related compared to isolates of different STs.

270

271 The antimicrobial resistance found are of relevance in a public health context,

272 particularly those to fluoroquinolones and macrolides (mainly ciprofloxacin and

273 erythromycin, respectively), but also tetracyclines and aminoglycosides. Quinolones

274 and macrolides are the antibiotic of choice to treat severe human *Campylobacter*

275 infections, whilst tetracyclines are used as an alternative treatment (Moore et al. 2006;

276 Butzler 2004) and aminoglycosides are recommended to treat bacteraemia caused by

277 *Campylobacter* (Kassa et al. 2007). Resistance to fluoroquinolones and macrolides is

278 quite common in poultry (Thakur 2010). High quinolone resistance in poultry has

279 previously been reported in Spain (Pérez-Boto et al. 2013; Melero et al. 2012), as well

280 as in other EU countries (Luber et al. 2003; Nobile et al. 2013). The high MIC values

281 detected here may be related to the presence of Thr86Ile in all of the isolates, which in

282 itself provides high resistance to quinolones (MIC >16 mg/L) (Ruiz et al. 1998). This

283 mutation is the most prevalent in clinical and veterinary isolates (Hormeño et al. 2016;

284 Butzler 2004). The high prevalence of isolates resistant to tetracyclines is similar to that

285 previously reported in poultry in Spain (Pérez-Boto et al. 2013; Melero et al. 2012;

286 Duarte et al. 2016). This resistance is mediated by the *tet(O)* gene which was detected in

287 all isolates showing tetracycline resistance. Besides the chromosomal location of this

288 gene, it has also been reported in plasmids (Avrain et al. 2004; Iovine 2013). In contrast,

289 resistance to aminoglycosides was diverse, with a considerably high resistance to

290 streptomycin (75%) and a much lower resistance to gentamicin (13%), in agreement

291 with Duarte et al. (2016) and Pérez-Boto et al. (2013). Gentamicin resistance in

292 *Campylobacter* spp. from poultry is a rare event all over European countries (Carreira et

293 al. 2012; De Jong et al. 2009; Pérez-Boto et al. 2013), probably because it is not used in

294 poultry production. The *aph(3′)-III* and *aadE* genes involved in aminoglycoside

295 resistance were identified in some strains. However, the finding of a high resistance to

296 streptomycin in some of the strains despite the absence of corresponding genes might be

297 due to the presence of undiscovered genes. Nevertheless, the present work was focused

298 on chromosomal genes, so whether these strains carried plasmids encoding

299 streptomycin resistance-genes cannot be ruled out and deserve further research (Iovine

300 2013). Over 50% of isolates were resistant to erythromycin and was more common

301 among *C. coli* than *C. jejuni*, similarly to what has been previously reported in poultry

302 in the EU (Duarte et al. 2016; Wimalarathna et al. 2013). Erythromycin resistance is

303 acquired through point mutations in domain V of the 23S rDNA at positions 2074 and

304 2075 (positions 2058 and 2059 in *E. coli* numbering) (Iovine 2013); the point mutation

305 A2075G which is the most prevalent in *Campylobacter* spp. and confers high-level

306 resistance to macrolides, was identified in three *C. coli* isolates. The overall resistances

307 detected here are those also common in food-producing animals in the EU, as reported

308 by EFSA (2015). Particularly, the pattern of resistance among *Campylobacter* isolates

309 was predominantly quinolones (ciprofloxacin and nalidixic acid) and tetracyclines,

310 whilst resistance to erythromycin and gentamicin was comparatively low. This is most

311 probably due to the frequent use of enrofloxacin (quinolone) and doxycycline

312 (tetracycline) in the studied farms.

313

314 Several virulence factors have been identified in *Campylobacter*, which include

315 flagella-mediated motility, bacterial adherence to intestinal mucosa, invasive capability

316 and the ability to produce toxins. *C. jejuni* isolates were positive for the presence of

317 most of the virulence genes analyzed related to these virulence factors. However, few

318 strains were negative for the *flaA* gene, whilst all were positive for the *flaB* gene. Those

319 adjacent genes encode for the protein flagellin which compose the flagellar filament, an

320 important colonization factor (Silva et al. 2011; Koolman et al. 2015). Isolates negative

321 for the *flaA* gene might have reduced motility and colonization ability (Neal-McKinney

322 et al. 2010). The gene *wlaN*, responsible for the expression of Guillain-Barré syndrome,

323 was detected with a low frequency, in agreement with other reports (Koolman et al.

324 2015; Datta et al. 2003; Talukder et al. 2008). In contrast, the multidrug CmeABC

325 efflux system, which has a role in antimicrobial resistance, was present in all but one

326 isolate that lacked the CmeB gene. The efflux system is common in *Campylobacter* and

327 consists of an external membrane protein (CmeC), a drug transporter in the internal

328 membrane (CmeB) and an external membrane protein (CmeA). They all form a

329 membrane channel that expels toxic substances from the cell (Lin et al. 2002). The *hipO*

330   gene, which is specific for *C. jejuni*, was not present in any of the *C. coli* isolates.

331   Besides this gene, and the *wlaN* gene, which is relatively rare, all virulence genes

332   analyzed were present in all *C. coli* strains. It is noteworthy to highlight that the *sodB*

333   gene involved in stress defense, and that was recently first reported in *C. coli* isolates by

334   Koolman et al. (2015), has also been found in all *C. coli* isolates in this study.

335

336   Altogether, the in-depth characterization of these poultry isolates contributes to the

337   understanding of *Campylobacter* epidemiology. WGS technology has become a fast and

338   affordable tool and may become a rapid and cost-effective approach to characterize

339   isolates from epidemiological studies (Llarena et al. 2017).

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481

1   **Figure legends**

2

3   **Figure 1.** Maximun-likelihood phylogenetic tree based on SNPs from the assembled

4   genomes and genotypic MLST types of the *Campylobacter* isolates. The tree was drawn

5   to scale, with branch lengths measured in the number of substitutions per site.

6   [a] Reference genomes for *C. jejuni* NCTC 11168 (Accession number: AL111168) and for

7   *C. coli* FB1 (Accession number: CP011015) were included in the analysis. The tree was

8   drawn to scale, with branch lengths measured in the number of substitutions per site.

9   [b] ST, sequence type; CC, clonal complex.

10

11   **Supplementary Fig. 1.** PFGE combined dendrogram of *Sma*I and *Kpn*I patterns of *C.*

12   *jejuni* and *C. coli* isolates.

13

14   **Supplementary Fig. 2.** Heatmap representing the SNPs between the genomes.

15

Isolates	Species	Quinolones			Tetracycline		Aminoglycosides					Macrolide		Efflux pump CmeA,B,C
		Nal [a]	Ci	R-mech [c] Thr86Ile	Tc	R-mech *tet(O)*	Sm	R-mech *aphA(3´)*	R-mech *aadE*	Gm	R-mech aphA(3´)	Ery	R-mech 23S rDNA	
A1	*C. coli*	R (> 64) [b]	R (> 8)	+	S (0,5)		R (32)	+		R (2)	+	R (32)	+	+ + +
A2	*C. jejuni*	R (32)	R (> 8)	+	S (0,5)		S (0,5)			S (0,12)		R (32)		+ + +
B3	*C. jejuni*	R (> 64)	R (> 8)	+	R (> 16)	+	R (32)			S (0,12)		R (> 64)		+ + +
B4	*C. jejuni*	R (32)	R (0,5)	+	R (> 16)	+	R (8)			S (0,12)		S (1)		+ + +
B5	*C. jejuni*	R (> 64)	R (> 8)	+	R (> 16)	+	R (> 64)	+	+	R (2)	+	S (0,5)		+ + +
B6	*C. jejuni*	R (> 64)	R (> 8)	+	R (> 16)	+	R (> 64)			S (1)		S (1)		+ + +
C7	*C. coli*	R (> 64)	R (4)	+	R (2)	+	R (32)			S (0,12)		R (> 64)	+	+ + +
C8	*C. coli*	R (> 64)	R (4)	+	R (> 16)	+	R (> 64)			S (0,25)		R (> 64)	+	+ + +
C9	*C. jejuni*	R (32)	R (4)	+	R (> 16)	+	S (0,5)			S (0,12)		S (1)		+ + +
C10	*C. jejuni*	R (> 64)	R (> 8)	+	R (> 16)	+	R (> 64)			S (0,12)		R (> 64)		+ + +
D11	*C. jejuni*	R (> 64)	R (> 8)	+	R (> 16)	+	S (0,5)			S (0,12)		S (1)		+ + +
D12	*C. jejuni*	R (> 64)	R (> 8)	+	R (> 16)	+	R (> 64)			S (1)		R (> 64)		+ + +
E13	*C. jejuni*	R (> 64)	R (4)	+	R (> 16)	+	S (0,5)			S (0,12)		S (1)		+ + +
E14	*C. jejuni*	R (> 64)	R (> 8)	+	R (> 16)	+	R (> 64)			S (0,12)		R (8)		+ +
E15	*C. jejuni*	R (> 64)	R (> 8)	+	S (0,5)		R (16)			S (0,25)		S (1)		+ + +
E16	*C. coli*	R (16)	R (> 8)	+	R (> 16)	+	R (4)			S (0,12)		R (> 64)		+ + +
Total		**100%**	**100%**		**81%**		**75%**			**13%**		**56%**		

[a] Nal: Nalidixic acid, Ci: Ciprofloxacin, Tc: Tetracycline, Sm: Streptomycin, Gm: Gentamicin and Ery: Erythromycin.

[b] Interpretation of MIC values for *C. jejuni* epidemiological cut-off values: Nal (R $\geq$ 16 mg/L); Ci (R $\geq$ 0,5 mg/L); Tc (R $\geq$ 1 mg/L); Sm (R $\geq$ 4 mg/L); Gm (R $\geq$ 2 mg/L) and Ery (R $\geq$ 4 mg/L). Interpretation for *C. coli* epidemiological cut-off values: Nal (R $\geq$ 16 mg/L); Ci (R $\geq$ 0,5 mg/L); Tc (R $\geq$ 2 mg/L); Sm (R $\geq$ 4 mg/L); Gm (R $\geq$ 2 mg/L) and Ery (R $\geq$ 8 mg/L).

[c] R-mech: resistance mechanism. Thr86Ile: point mutations in the subunit A of the DNA gyrase gene; tet(O), aphA(3') and aadE: presence of these genes; 23S rDNA: point mutation on this region of the genome.

TABLE 2. DISTRIBUTION OF VIRULENCE GENES IN *C. JEJUNI* AND *C. COLI* ISOLATES*

Isolates	Species	Motility[a]		GBS	Hippuricase gene	Multidrug and bile resistance			Iron uptake	
		flaA	*flaB*	*wlaN*	*hipO*	*cmeA*	*cmeB*	*cmeC*	*cfrA*	*fur*
A1	*C. coli*	+	+			+	+	+	+	+
A2	*C. jejuni*		+		+	+	+	+	+	+
B3	*C. jejuni*	+	+		+	+	+	+	+	+
B4	*C. jejuni*	+	+		+	+	+	+	+	+
B5	*C. jejuni*	+	+		+	+	+	+	+	+
B6	*C. jejuni*		+		+	+	+	+	+	+
C7	*C. coli*	+	+			+	+	+	+	+
C8	*C. coli*	+	+			+	+	+	+	+
C9	*C. jejuni*	+	+	+	+	+	+	+	+	+
C10	*C. jejuni*		+		+	+	+	+	+	+
D11	*C. jejuni*	+	+		+	+	+	+	+	+
D12	*C. jejuni*		+		+	+	+	+	+	+
E13	*C. jejuni*	+	+	+	+	+	+	+	+	+
E14	*C. jejuni*		+		+	+		+	+	+
E15	*C. jejuni*	+	+		+	+	+	+		+
E16	*C. coli*	+	+			+	+	+	+	+
Total		11	16	2	12	16	15	16	15	16

*The presence of genes related to motility (*flhA, flhB, flgB, flgE, fliM, fliY*); chemotaxis (*cheA, cheB, cheR, cheW, cheY*); adhesion (*cadF, dnaJ, pdlA, racR*); capsule *(kpsM, waaF)*; Invasion (*iamA, ciaB, ceuE*); Cytolethal distending toxin (*cdta, cdtB, cdtC*); Stress response and survival (*katA, sodB*), was also confirmed in all the isolates, (data not shown to facilitate the reading). [a]The results for fla genes was confirmed by PCR.

SUPPLEMENTARY TABLE 1. ASSEMBLY METRICS

Isolates	Species	N50	Assembly size	Nº contigs	Coverage
A1	*C. coli*	52.853	1.746.294	133	49.73
A2	*C. jejuni*	102.764	1.713.572	45	114.7
B3	*C. jejuni*	59.250	1.730.328	100	85.37
B4	*C. jejuni*	106.203	1.748.765	34	72.99
B5	*C. jejuni*	66.683	1.688.181	130	82.47
B6	*C. jejuni*	221.977	1.688.205	36	100.3
C7	*C. coli*	168.863	1.638.379	62	75.94
C8	*C. coli*	166.289	1.746.251	58	127.2
C9	*C. jejuni*	91.129	1.723.771	55	89.97
C10	*C. jejuni*	162.254	1.690.078	30	120.6
D11	*C. jejuni*	148.283	1.675.334	53	86.66
D12	*C. jejuni*	91.211	1.705.710	68	117.6
E13	*C. jejuni*	79.604	1.677.193	72	104.4
E14	*C. jejuni*	83.132	1.811.001	97	78.98
E15	*C. jejuni*	59.658	1.591.333	93	65.64
E16	*C. coli*	79.519	1.687.906	49	100.6

SUPPLEMENTARY TABLE 2. VIRULENCE ASSOCIATED GENES CONSIDERED IN THIS STUDY AND THEIR CORRESPONDING LOCUS TAG

Genes	Locus tag	
	C. jejuni	*C. coli*
flaA	Cj0887c	VC76_04395
flaB	Cj0887c	VC76_03535
flhA	Cj0882c	VC76_04415
flhB	Cj0335	VC76_01775
flgB	Cj0526c	VC76_02755
flgE	Cj1729c	VC76_08610
fliM	Cj0060c	VC76_00300
fliY	Cj0059c	VC76_03960
cheA	Cj0284c	VC76_01475
cheB	Cj0924c	VC76_04655
cheR	Cj0923c	VC76_04650
cheW	Cj0283c	VC76_01470
cheY	Cj1118c	VC76_05495
cadF	Cj1478c	VC76_07295
dnaJ	Cj0954c	VC76_03025
pldA	Cj1351	VC76_06765
racR	Cj1261	VC76_03020"
cdtA	Cj0079c	VC76_01515
cdtB	Cj0078c	VC76_01510
cdtC	Cj0077c	VC76_01505
wlaN	Cj1139c	Absent
iamA	Cj1647	VC76_00545
ciaB	Cj0914c	VC76_04610
ceuE	Cj1355	VC76_06790
cmeA	Cj0365c	VC76_01930
cmeB	Cj0366c	VC76_01925
cmeC	Cj0365c	VC76_07315
katA	Cj1385	VC76_06950
sodB	Cj0169	VC76_07880
cfrA	Cj0755	VC76_03645
fur	Cj0400	VC76_02095
kpsM	Cj1448c	VC76_07145
waaF	Cj1148	VC76_05560
hipO	Cj0985c	Absent

SUPPLEMENTARY TABLE 3. HOMOLOGY ANALYSES OF EACH STUDIED GENE OF THE REFERENCE STRAINS (*C. jejuni* NCTC 11168 and *C. coli* FB1) WITH RESPECT TO THE CORRESPONDING GENES IN THE TESTED ISOLATES

Isolates	Species	Motility							
		flaA		*flaB*		*flhA*		*flhB*	
		% Identity	Query/HSP length	% Identity	Query/HSP length	% Identity	Query/HSP length	% Identity	Query/HSP length
A1	*C. coli*	95.12	2253/2253	99.47	756/756	99.91	2178/2178	100	1089/1089
A2	*C. jejuni*					100	2175/2175	100	1089/1089
B3	*C. jejuni*					99.72	2175/2175	99.82	1089/1089
B4	*C. jejuni*	98.59	1133/1719	98.15	1133/1719	99.95	2175/2175	100	1089/1089
B5	*C. jejuni*	97.07	1261/1719			100	2175/2175	98.16	1086/1089
B6	*C. jejuni*					99.72	2175/2175	99.82	1089/1089
C7	*C. coli*	97.96	2253/2253	100	756/756	100	2178/2178	100	1089/1089
C8	*C. coli*	95.12	2253/2253	99.47	756/756	99.91	2178/2179	100	1089/1089
C9	*C. jejuni*	99.38	1133/1719	99.82	1113/1719	100	2175/2175	99.91	1089/1089
C10	*C. jejuni*					99.72	2175/2175	99.82	1089/1089
D11	*C. jejuni*	92.64	1263/1719			99.95	2175/2175	100	1089/1089
D12	*C. jejuni*					99.95	2175/2175	100	1089/1089
E13	*C. jejuni*	98.73	1261/1719			98.73	1261/1719	99.91	1089/1089
E14	*C. jejuni*					99.91	2175/2175	99.72	1089/1089
E15	*C. jejuni*	91.77	1325/1719			91.77	1325/1719	99.82	1089/1089
E16	*C. coli*	96.40	2253/2253	100	756/756	100	2178/2178	100	1089/1089

SUPPLEMENTARY TABLE 3 CONTINUED.

Isolates	Species	Motility							
		flgB		*flgE*		*fliM*		*fliY*	
		% Identity	Query/HSP length	% Identity	Query/HSP length	% Identity	Query/HSP length	% Identity	Query/HSP length
A1	*C. coli*	100	432/432	95.91	1638/1638	92.04	1080/1080	99.74	771/771
A2	*C. jejuni*	97.22	432/432	99.82	1638/1638	99.63	1080/1080	99.64	843/843
B3	*C. jejuni*	96.76	432/432	99.88	1638/1638	100	1080/1080	100	843/843
B4	*C. jejuni*	100	432/432	99.82	1638/1638	98.43	1080/1080	99.76	843/843
B5	*C. jejuni*	100	432/432	100	1638/1638	98.43	1080/1080	99.76	843/843
B6	*C. jejuni*	96.76	432/432	98.78	1638/1638	92.41	1080/1080	100	843/843
C7	*C. coli*	100	432/432	98.78	1638/1638	92.41	1080/1080	99.87	771/771
C8	*C. coli*	100	432/432	95.91	1638/1638	90.37	1080/1080	99.74	771/771
C9	*C. jejuni*	100	432/432	100	1638/1638	98.43	1080/1080	99.76	843/843
C10	*C. jejuni*	96.76	432/432	99.88	1638/1638	100	1080/1080	100	843/843
D11	*C. jejuni*	97.22	432/432	99.88	1638/1638	98.43	1080/1080	99.64	843/843
D12	*C. jejuni*	100	432/432	99.82	1638/1638	98.43	1080/1080	99.76	843/843
E13	*C. jejuni*	97.22	432/432	99.88	1638/1638	98.43	1080/1080	99.64	843/843
E14	*C. jejuni*	97.22	432/432	99.88	1638/1638	98.06	1080/1080	99.53	843/843
E15	*C. jejuni*	99.77	432/432	99.88	1638/1638	97.69	1080/1080	99.56	843/843
E16	*C. coli*	100	432/432	98.41	1638/1638	92.41	1080/1080	99.74	771/771

SUPPLEMENTARY TABLE 3 CONTINUED.

Isolates	Species	Chemotaxis									
		cheA		cheB		cheR		cheW		cheY	
		% Identity	Query/HSP length	% Identity	Query/HSP length	% Identity	Query/HSP length	% Identity	Query/HSP length	% Identity	Query/HSP length
A1	C. coli	99.96	2295/2295	100	555/555	99.87	798/798	100	522/522	100	957/957
A2	C. jejuni	99.96	2310/2310	99.82	555/555	100	798/798	100	522/522	99.75	393/393
B3	C. jejuni	99.31	2310/2310	99.82	554/555	99.75	798/798	99.81	522/522	99.75	393/393
B4	C. jejuni	99.48	2310/2310	100	555/555	100	798/798	100	522/522	99.75	393/393
B5	C. jejuni	99.96	2310/2310	100	555/555	100	798/798	100	522/522	100	393/393
B6	C. jejuni	99.31	2310/2310	99.82	554/555	99.75	798/798	99.81	522/522	99.75	393/393
C7	C. coli	100	2295/2295	100	555/555	99.75	798/798	100	522/522	100	957/957
C8	C. coli	99.96	2295/2295	100	555/555	99.87	798/798	100	522/522	100	957/957
C9	C. jejuni	99.96	2310/2310	100	555/555	100	798/798	100	522/522	100	393/393
C10	C. jejuni	99.31	2310/2310	99.82	554/555	99.75	798/798	99.81	522/522	99.75	393/393
D11	C. jejuni	99.96	2310/2310	100	555/555	100	798/798	100	522/522	99.75	393/393
D12	C. jejuni	99.48	2310/2310	100	555/555	100	798/798	100	522/522	99.75	393/393
E13	C. jejuni	99.96	2310/2310	100	555/555	100	798/798	100	522/522	99.75	393/393
E14	C. jejuni	99.96	2310/2310	100	555/555	99.87	798/798	100	522/522	99.75	393/393
E15	C. jejuni	98.01	2310/2310	98.74	554/555	99.11	798/798	99.23	522/522	99.49	393/393
E16	C. coli	100	2295/2295	100	555/555	99.62	798/798	100	522/522	100	957/957

SUPPLEMENTARY TABLE 3 CONTINUED.

Isolates	Species	Adhesion							
		cadF		dnaJ		pldA		racR	
		% Identity	Query/HSP length	% Identity	Query/HSP length	% Identity	Query/HSP length	% Identity	Query/HSP length
A1	C. coli	99.60	999/999	98.22	1125/1125	100	996/996	99.85	672/672
A2	C. jejuni	100	960/960	100	1122/1122	99.98	990/990	100	672/672
B3	C. jejuni	100	960/960	99.73	1122/1122	98.79	990/990	100	672/672
B4	C. jejuni	100	960/960	99.29	1122/1122	98.69	990/990	99.85	668/672
B5	C. jejuni	100	960/960	100	1122/1122	100	990/990	100	672/672
B6	C. jejuni	100	960/960	99.73	1122/1122	98.79	990/990	100	672/672
C7	C. coli	99.70	999/999	99.73	1125/1125	99.60	996/996	100	672/672
C8	C. coli	99.60	999/999	98.67	1125/1125	100	996/996	99.85	668/672
C9	C. jejuni	100	960/960	98.04	1125/1125	100	990/990	99.55	672/672
C10	C. jejuni	100	960/960	99.73	1122/1122	98.79	990/990	100	672/672
D11	C. jejuni	100	960/960	100	1122/1122	100	990/990	100	672/672
D12	C. jejuni	100	960/960	99.29	1122/1122	98.69	990/990	99.85	672/672
E13	C. jejuni	100	960/960	100	1122/1122	99.90	990/990	100	672/672
E14	C. jejuni	99.58	960/960	98.76	1122/1122	99.09	990/990	99.70	672/672
E15	C. jejuni	99.06	960/960	100	1122/1122	98.59	990/990	100	672/672
E16	C. coli	99.70	999/999	99.20	1125/1125	99.20	996/996	100	672/672

	Isolates	Species	ST[b]	CC[b]
	E15	*C. jejuni*	45	45
	E14	*C. jejuni*	824	257
	B6	*C. jejuni*	257	257
	C10	*C. jejuni*	257	257
	B3	*C. jejuni*	257	257
	D12	*C. jejuni*	572	206
	B4	*C. jejuni*	572	206
	A2	*C. jejuni*	122	206
	D11	*C. jejuni*	50	21
	E13	*C. jejuni*	8586	21
	B5	*C. jejuni*	21	21
	NCTC 11168[a]	*C. jejuni*		
	C9	*C. jejuni*	21	21
	FB1[a]	*C. coli*		
	E16	*C. coli*	8578	828
	C7	*C. coli*	827	828
	A1	*C. coli*	854	828
	C8	*C. coli*	854	828
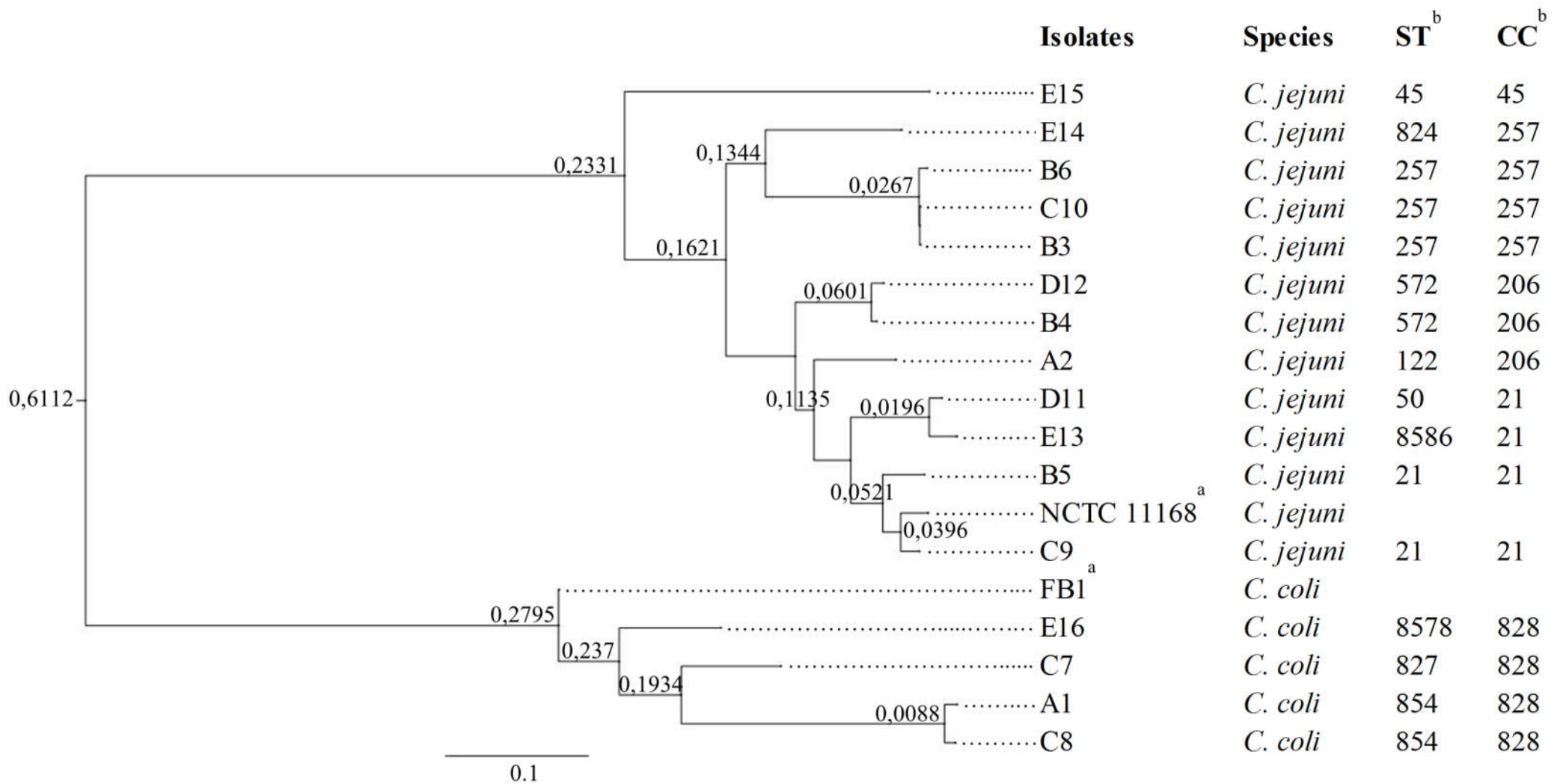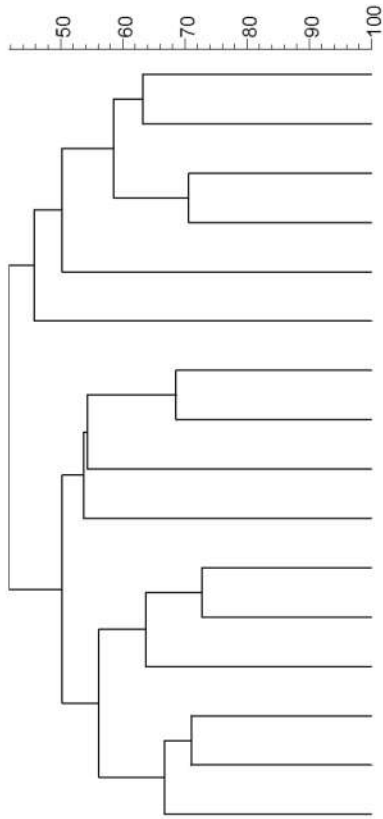
SmaI	KpnI	Isolates	Species	Year
		E16	*C. coli*	2013
		A1	*C. coli*	2011
		C8	*C. coli*	2012
		C7	*C. coli*	2012
		C9	*C. jejuni*	2013
		A2	*C. jejuni*	2012
		B4	*C. jejuni*	2011
		D12	*C. jejuni*	2011
		E15	*C. jejuni*	2013
		B5	*C. jejuni*	2012
		E13	*C. jejuni*	2011
		E14	*C. jejuni*	2011
		D11	*C. jejuni*	2011
		B6	*C. jejuni*	2013
		C10	*C. jejuni*	2013
		B3	*C. jejuni*	2011

SNP counts between genomes

6   8420

Not yet implemented

0   100