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1 **Genome-wide identification of host-segregating SNPs for source attribution of clinical *Campylobacter coli***

2 **isolates**

3

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16

17 Running title: Source attribution for *Campylobacter coli*

18

19 Keywords: *Campylobacter coli*, SNP, source attribution, genomics, genotyping.

20 **ABSTRACT**

21 *Campylobacter* is among the most common causes of gastroenteritis worldwide. *Campylobacter*  
22 *jejuni* and *Campylobacter coli* are the most common species causing human-disease. DNA-sequence-  
23 based methods for strain characterization have focussed largely on *C. jejuni*, responsible for 80-90%  
24 of infections, meaning that *C. coli* epidemiology has lagged behind. Here we have analyzed the  
25 genome of 450 *C. coli* isolates to determine genetic markers that can discriminate isolates sampled  
26 from 3 major reservoir hosts (chickens, cattle and pigs). These markers were then applied to identify  
27 the source of infection of 147 *C. coli* from French clinical cases. Using STRUCTURE software, 259  
28 potential host-segregating markers were revealed by probabilistic characterization of SNP frequency  
29 variation in strain collections from three different hosts. These SNPs were found in 41 genes or  
30 intergenic regions, mostly coding for proteins involved in motility and membrane functions. Source  
31 attribution of clinical isolates based on the differential presence of these markers confirmed chicken  
32 as the most common source of *C. coli* infection in France.

33

34 **IMPORTANCE** Genome-wide and source attribution studies based on *Campylobacter* species have  
35 shown their importance for the understanding of foodborne infections. Although the use of MLST  
36 based on 7 genes from *C. jejuni* is a powerful method to structure populations, when applied to *C.*  
37 *coli* results have not clearly demonstrated their robustness. Therefore, we aim here to provide more  
38 accurate data based on the identification of single-nucleotide polymorphisms. Results from this  
39 study reveal an important number of host-segregating SNPs, found in proteins implied in motility,  
40 membrane functions or DNA repair systems. These findings offer new interesting opportunities for  
41 further study on *C. coli* adaptation to its environment. Additionally, the results demonstrate that  
42 poultry is potentially the main reservoir of *C. coli* in France.

43 **INTRODUCTION**

44 *Campylobacter* is the leading cause of bacterial gastroenteritis worldwide (1), with around 800,000  
45 campylobacteriosis cases in the USA (2) and 200,000 in the European Union (3) each year.

46 Demographic, dietary and surveillance programs variations have made it difficult to generalise  
47 understanding of *Campylobacter* epidemiology to all countries. For example, while there are an  
48 estimated 68,000 foodborne infections every year in France (4), the number attributable to  
49 *Campylobacter* is not clearly defined, and there are questions about the relative importance of  
50 different *Campylobacter* species (5) (6) (7).

51

52 *C. jejuni* and *C. coli* are part of the commensal microbiota of many bird and animal species (8).

53 Human infection typically occurs via consumption of contaminated meat - especially chicken (9) (10)  
54 (11), water or direct contact with animals (livestock farming). Infection is usually self-limiting with  
55 mild symptoms including abdominal cramps, diarrhoea and fever. However, more severe symptoms  
56 such as bloodstream infections and vascular disease can occur, particularly at the extreme ages of  
57 life, in immunosuppressed, diabetic or cancer patients, and in rare cases, post-infectious  
58 complications include Guillain–Barré syndrome (12) and irritable bowel syndrome (13). Prolonged or  
59 severe campylobacteriosis can require the administration of macrolide (azithromycin) or quinolone  
60 (ciprofloxacin) (14) (15) antibiotics but increasing resistance, particularly among *C. coli* isolates (16),  
61 is reducing treatment options.

62

63 *C. coli* is responsible for an increasing number of infections, accounting for approximately 15% of all  
64 campylobacteriosis cases (6). While much research focuses on *C. jejuni*, accounting for about 85% of  
65 cases, there are proportional differences between countries potentially reflecting variations in diet

66 (17) and host source (18) (19). European studies have typically associated *C. coli* with pigs and sheep  
67 (5) (20) (21). However, intensive agricultural practices in recent decades have dramatically changed  
68 the distribution of livestock species on earth creating opportunities for host transitions (22). This has  
69 likely driven changes to the natural host associations of both *C. jejuni* and *C. coli* which are regularly  
70 isolated from cattle and chickens (9). This host melting-pot has also dramatically affected the  
71 evolution of livestock associated *C. coli* leading to the emergence of a dominant disease-causing *C.*  
72 *coli* lineage, the ST-828 clonal complex (*CC-828*) (23), that has a mosaic genome with over 10% of  
73 the genes having been acquired from *C. jejuni* by horizontal gene transfer (24) (25) (26). This  
74 genome plasticity is particularly of concern for *C. coli* which acquires antimicrobial resistance genes  
75 more easily than *C. jejuni* (14) (16).

76

77 Genotyping methods such as multilocus sequence typing (MLST) (27) (28) have improved our  
78 understanding of *Campylobacter* population structure, revealing host-specialist and host-generalist  
79 lineages (29). This host association has underpinned the development of methods that  
80 quantitatively attribute the source of human infections (9) (11). However, rapid host-switching by  
81 host generalist *Campylobacter*, including *C. coli CC-828*, can often confound these methods because,  
82 for some lineages, strains associated with one host source can be found in another (22) (30). The  
83 adoption of whole genome sequencing techniques and availability of curated genome databases  
84 (31) have allowed the incorporation of a broader number of host-segregating epidemiological  
85 markers in source attribution methods (32) (33). This additional genome information has increased  
86 the resolution allowing attribution of invasive/non-invasive strains from poultry (34) as well as  
87 geographical attribution of UK/USA isolates (19). However, almost all studies focussed exclusively on  
88 *C. jejuni* (35), and no study aimed to specifically identify host-segregating markers in *C. coli* genomes.

89

90 In this study, we analyzed 450 *C. coli* genomes from public databases with defined sampling sources  
91 including chickens, cattle and pigs. Using comparative genomics approaches we: (i) tested the ability  
92 of traditional MLST-based methods to determine the source of *C. coli* with isolates from known  
93 source reservoirs; (ii) identified host-segregating SNPs in *C. coli* genomes; (iii) determined the  
94 relative contribution of different *C. coli* infection sources in France. MLST was found to be a good  
95 proxy for more complex whole genome SNP-based analysis, showing similar power for segregating  
96 isolates from cattle host. However, additional discrimination of isolates from chicken and pig hosts  
97 was achieved by identifying genome-wide host-segregating SNPs. In the final probabilistic model,  
98 using 259 host-segregating SNPs, chicken was found to be the most common source of *C. coli*  
99 infection in France.

100 **RESULTS**

101

102 **CC-828 isolates segregate by host**

103 From all 3 datasets, Dataset S1, S2 and S3 (cf. material and methods), nearly all isolates belonged to  
104 the clonal complex -828 (780 isolates out of 900). The second most common clonal complex  
105 identified was *ST-1150* (26) with four isolates, sampled from chicken. From the allelic profiles  
106 minimum spanning tree, 3 clusters can be identified corresponding to the source of isolation (Figure  
107 1). Cattle isolates clustered together, with 162 isolates (64.8% of all cattle isolates) assigned to *ST-*  
108 *1068* (36). Chicken and pig isolates belonged to 78 and 83 sequence types respectively (contrary to  
109 cattle with 27 different sequence types), with 24.2% isolates belonging to *STs* -828, -829, -825, -854,  
110 and -1119. Furthermore, 40.1% of all clinical isolates belonged to *STs* -825, -827, -832 and -860.  
111 Initial evidence for a role for chicken as a reservoir for human infection was provided by the  
112 clustering of clinical isolates together with isolates from chicken on the phylogenetic tree. The  
113 second tree constructed using maximum-likelihood approach from concatenated SNPs sequences  
114 revealed distinctive partitioning of isolates according to source (Figure 2). *C. coli* isolated from cattle  
115 constitute a very distinct cluster; 168 isolates (67.2% of all cattle isolates) are located at the bottom  
116 of the tree and belonged to *ST-1068*. Distances were also shorter within cattle population compared  
117 to chicken and pig isolates where more variability was observed within both clades. While many  
118 clinical isolates clustered among chicken isolates, six clinical isolates were found along a long branch  
119 of the chicken's clade - these isolates were interestingly attributed to pig using STRUCTURE (below).

120

121

122 **Host-segregating SNPs differentiate *C. coli* isolated from different hosts**

123 Putative host-segregating SNPs were identified by aligning all 450 isolates selected for marker  
124 determination against three *C. coli* reference genomes. Alignment of isolates against the OR12 *C. coli*  
125 reference strain identified 283,320 variant sites. In order to remove weakly discriminating  
126 polymorphisms, SNP versions represented in more than two thirds of all isolates were filtered,  
127 leaving 26,131 variant sites. Similar alignment and filtering performed against the HC2-48 strain  
128 resulted in 202,111 variants, filtered to 24,395; and alignment against the ZV1224 reference  
129 identified 242,574 SNPs, which were filtered to 20,827. Host-segregating SNPs were identified by  
130 performing source attribution tests using each variant individually and all 450 isolates. SNPs with at  
131 least 70 % accuracy for at least one source in the self-attribution test included 43, 183 and 33 from  
132 each alignment with the OR12, HC2-48 and ZV1224 reference strains, respectively (Table 1). Most of  
133 the self-attribution tests showed rates fluctuating between 30% and 40% (Figure 3) (51.2%, 50.5%,  
134 48% of all variants for the chicken, cattle and pig variants, respectively); 33% indicates a complete  
135 inability to differentiate 3 individuals. In total, 259 host-segregating SNPs from 41 nucleotide  
136 sequences were carried forward for further analyses.

137

138 To contextualize host-segregating SNPs within genes, Blast-x annotation identified 32 coding regions  
139 for known proteins, 5 hypothetical proteins as well as 4 intergenic regions (Suppl Table S1). Several  
140 SNPs (n=27) were found in proteins involved in motility, which plays an important role in bacterial  
141 host adaptation: 12 and 4 SNPs in flagellar proteins *FliK* (with 2 SNPs in its basal-body rod  
142 modification protein *FlgD*) and *FliD* respectively, known to modulate flagellar hook length (37) and  
143 to act as an immunodominant protein (38); 5 SNPs from methyl-accepting chemotaxis proteins (TLP-  
144 like protein (39)) or intergenic regions before methyl-accepting chemotaxis proteins; as well as 4



145 SNPs in one aerotaxis receptor belonging to *CetC*, a protein involved in regulating energy taxis (40).  
146 Another protein involved in bacterial adaptation to its environment has also been identified from  
147 OR12 chicken reference (3 SNPs): *SbmA* (41), a peptide antibiotic transporter described in many  
148 gram-negative bacteria. SNPs were also found in proteins involved in metabolism and membrane  
149 functions: 3 SNPs from an histidine kinase, 5 SNPs from a single-domain globin protein, known to  
150 play a role against NO and nitrosative stress (42), and a *LamB/YcsF* family protein with 5 SNPs. Two  
151 phosphate-binding proteins showed the presence of one SNP from OR12 chicken reference variant  
152 calling as well as one SNP from ZV1224 pig reference. Proteins involved in DNA activities have also  
153 been identified, with a total of 56 SNPs: DNA recombination/repair protein *RecA*, excinuclease ABC  
154 subunit C (*UvrC*) (43), two restriction endonucleases from HC2-48 and ZV1224 references, and one  
155 transcriptional regulator. Two hypothetical proteins from OR12 and ZV1224 with 11 and 8 host-  
156 segregating SNPs respectively have been found to be the same protein: its domains and amino-acid  
157 sequence depending the source should be further investigated. Finally, a total of 110 SNPs were  
158 within 2 hypothetical proteins (from the HC2-48 cattle reference), which reflected highly variable  
159 and isolate-specific regions, and should not be taken into account.

160

#### 161 **Genome-wide host-segregating SNPs provide more accurate source attribution than MLST alleles**

162 The degree of SNP segregation among isolates from different hosts, and hence the potential as  
163 marker for source attribution using STRUCTURE, was quantified. Self-attributions of chicken and pig  
164 isolates within the marker-determination dataset were consistently correct (Table 2). Using 43 SNPs  
165 detected from OR12 alignment as host-segregating markers allowed an average correct self-  
166 attribution of 88.35% (s.d.  $\pm$  6.2%), 63.75% (s.d.  $\pm$  9.2%) and 96.2% (s.d.  $\pm$  4.1%) for chickens, cattle  
167 and pigs respectively. Using 183 SNPs from HC2-48 alignment correct self-attribution was achieved

168 for chicken, cattle and pig isolates with 91.05% (s.d.  $\pm$  5.7%), 75% (s.d.  $\pm$  9.7%) and 42.45% (s.d.  $\pm$   
169 18.7%) accuracy respectively, and 74.95% (s.d.  $\pm$  13.9%), 19.65% (s.d.  $\pm$  10.1%) and 94.65% (s.d.  $\pm$   
170 5.2%) for the 33 SNPs from ZV1224 alignment. Low self-attribution rate of cattle isolates using SNPs  
171 from pig reference was observed: these isolates were not correctly attributed and were considered  
172 as 50% chicken and 50% pig. When using all the SNPs simultaneously (n=259), correct self-  
173 attribution showed average scores of 91.95% (s.d.  $\pm$  5.86%), 77% (s.d.  $\pm$  8.65%) and 95.25% (s.d.  $\pm$   
174 4.4%) for chickens, cattle and pigs respectively. This is a considerable improvement of self-  
175 attribution using the 7 MLST genes which returned average scores of 73.6% (s.d.  $\pm$  9.1%), 76.8% (s.d.  
176  $\pm$  9.4%) and 74.4% (s.d.  $\pm$  9.5%) for chickens, cattle and pigs respectively. Source attribution of cattle  
177 *C. coli* isolates of marker-determination dataset was similar between the two types of markers  
178 (genotype or allele) whereas SNPs performed significantly better for chicken and pig populations  
179 than the 7 MLST genes. Finally, the discriminatory power of host-segregating SNPs and MLST genes  
180 was evaluated performing source re-attribution of 299 *C. coli* isolates from the validation dataset.  
181 SNPs showed correct re-attribution proportions of 96.2% (s.d.  $\pm$  1.03%), 84% (s.d.  $\pm$  0%) and 89%  
182 (s.d.  $\pm$  0%), and MLST genes scores of 87% (s.d.  $\pm$  0%), 81% (s.d.  $\pm$  0%) and 65% (s.d.  $\pm$  0%) for  
183 chicken, cattle and pig populations, respectively (Figure 4). Overall, SNPs were able to better re-  
184 attribute *C. coli* marker-determination and validation isolates to their source than MLST genes, more  
185 specifically for chickens and pig populations.

186

#### 187 **Chickens are a major source of *C. coli* infection in France**

188 Source attribution of clinical isolates was performed using MLST alleles and all host-segregating SNPs  
189 with correct self-attribution >70% (n=259) in the marker-determination and training dataset using  
190 STRUCTURE (Figure 5). Using MLST genes, 89 clinical isolates (60.5%) were attributed to chickens, 13

191 to cattle (9%), 6 to pigs (4%) and 39 clinical isolates (26.5%) showed attribution scores lower than  
192 70% and were therefore considered as "inconclusive attributions". Inconclusive attributions  
193 specifically concern 3 commonly found sequence types: *STs* -827, -1055 and -1595, representing  
194 48.7% of inconclusive attributions (n=19). In contrast, using the 259 SNPs, 138 isolates (94%) were  
195 attributed to chickens, 9 to pigs (6%) (with an average of source probability equal to 100%) and none  
196 to the cattle population. Therefore whatever the approach (MLST or SNPs), a large proportion of *C.*  
197 *coli* clinical isolates were attributed to chickens. However, the attribution scores were more variable  
198 with MLST (on average around 80%) whereas for the genome-wide host-segregating SNPs, the  
199 clinical isolates were more efficiently attributed to their infection source (Table 3).

200 **DISCUSSION**

201

202 The increasing availability of bacterial isolate genome collections and bioinformatics tools for large-  
203 scale analysis provides significant opportunities for understanding the genetic basis of phenotype  
204 variation in bacteria. Host adaptation is a key feature in the epidemiology of zoonotic pathogens  
205 (44), such as *Campylobacter*, and there has been considerable effort to identify host-associated  
206 genetic variation that can improve understanding of the evolution and origin of infecting strains.

207 Comparative genomic analyses have revealed core and accessory genome variation within *C. jejuni*  
208 that is associated with a given host/environment (45) (46) and this has been used to identify  
209 genome-wide host-segregating markers for source attribution (32). However, little comparable work  
210 has focussed on *C. coli*.

211

212 Genetic variation in bacterial genomes not only reflects adaptation to different hosts/sources but  
213 also temporal and geographic variation among sample collections (19). Some studies avoid the  
214 potential confounding effect of phylogeographic variation by using national isolate collections: for  
215 example, *Campylobacter* attribution studies performed in Scotland (24) (47), Switzerland (48), New  
216 Zealand (49) and Germany (17). This has been informative for understanding the source of human  
217 infection but, because of the strong segregation of genetic variation by host (18), it remains possible  
218 that collections from multiple countries could be combined to create international isolate  
219 collections. This would consolidate research effort and provide the large genome collections  
220 necessary for probabilistic attribution models and potential to identify universal host-segregating  
221 markers.

222

223 Here we analyzed *C. coli* isolates from Europe and the USA using the conventional MLST method  
224 established by Dingle *et al.*, in 2001 (27) and specific host-segregating SNPs. A single clonal complex  
225 (CC-828) dominated among the isolates independently of source and geographical location,  
226 representing 780 isolates over 900. The predominance of CC-828 isolates among *C. coli* (66% - 81%  
227 of all isolates (17) (24) (36)) with the ST-1150 complex accounting for most of the remaining isolates  
228 (26), confounds efforts to identify host-association at the clonal complex level – that is possible for  
229 *C. jejuni* (18). However, within CC-828 there was evidence for sequence types that were more  
230 commonly isolated from particular hosts. For example, ST-829, ST-832, ST-825 and ST-860  
231 predominated among chicken isolates, ST-827 was more common in pigs and ST-1068 was nearly  
232 exclusive to cattle, consistent with previous studies (36) (50). Similar low diversity in cattle *C. coli*  
233 isolates has previously been described among ruminant isolates from Scotland (47). A weaker host-  
234 association signal, based upon MLST alleles, compared to *C. jejuni* has made it difficult to  
235 distinctively partition *C. coli* by source (49). However, genotype segregation in *C. coli* provided initial  
236 evidence that the genomes of these isolates would contain host-segregating genetic signatures.

237

238 Estimating the discriminating power of genetic markers can be performed by determining the  
239 probability that a given genetic element - such as a single mutation - will be found among isolates  
240 from a given host (self-attribution). As in previous studies (32) (33), we used STRUCTURE software  
241 and self-attribution to determine the predictive power of putative host-segregating markers.  
242 Moreover, a recent review (35) mentioned that MLST genes were used for self-attribution tests in 6  
243 studies for both *C. coli* and *C. jejuni* (11) (24) (32) (33) (48) (51). However, correct attribution rates  
244 for *C. coli* showed inconsistent results for chickens (63-95%), cattle (26-89%) and pigs (70-94%),  
245 suggesting that a SNP-based approach may be advantageous for source attribution of *C. coli*. In fact,

246 we showed here that SNPs as host-segregating markers provided more accurate results for chickens,  
247 cattle and pigs with 92% (s.d.  $\pm$  5.9%), 77% (s.d.  $\pm$  8.7%) and 95.3% (s.d.  $\pm$  4.4%) correct attribution  
248 rates, respectively. While the difficulty in precise self-attribution using MLST genes is undoubtedly  
249 linked to reduced resolution, as CC-828 isolates dominate among *C. coli* populations (23), the  
250 transmission of *C. coli* between different host species would also reduce the discriminatory power of  
251 source-specific markers potentially leading to incorrect source attribution (22). Adjusting for single  
252 mutation determination thus provided promising candidates for accurate source attribution of  
253 human *C. coli* isolates. Of 669,019 SNPs from the alignment of 450 genomes against 3 references,  
254 259 SNPs in genes associated with cell membrane (transporters, binding proteins), chemotaxis (*FliK*,  
255 *FlhD*, TLP-like protein), DNA activities (*RecA*, *UvrC*) or energy (*CetC*) functions were chosen for  
256 attributing 147 clinical *C. coli* isolates to source.

257

258 It is known that poultry are a major reservoir for human *C. jejuni* infection (8), with a ratio of 9:1 for  
259 *C. jejuni* and *C. coli*, respectively (36). Previous studies focussing on the source of *C. coli* infection  
260 have come to contrasting conclusions. In France, Sweden, the UK and the USA, the high prevalence  
261 of *C. coli* in pigs led to the assumption of the role of this reservoir in human infection (5) (20) (21), up  
262 to a ratio of 9:1 in favor of *C. coli* (36). However, in New Zealand, where human *C. coli* infection is  
263 also common, there is a low prevalence in pigs (49). Estimates of the relative contribution of  
264 different host sources to human infection varies among studies (11) (17) (24) (47) (48) (49) (52) with  
265 attribution to poultry (38-86%), ruminants (0-55%) and pigs (1-32%) all being implicated. With the  
266 exception of two studies, including rural populations in Scotland and New Zealand, that largely  
267 attribute human *C. coli* infections to sheep (47) and ruminants (49), source attribution studies  
268 typically assign a principal role for poultry in human infection.

269

270 It is likely that there are differences in the major reservoirs of *C. coli* infection in different countries  
271 but quantifying this requires accurate estimation. Estimates based upon MLST loci provided source  
272 probabilities with some uncertainty. Specifically, although approximately 40% of the 147 French  
273 clinical isolates sampled in this study were clearly attributed (>90% probability, Figure 5), the  
274 remaining isolates showed variable scores with many attributed with <60% probability. Overall,  
275 MLST-allele-based analyses did assign chicken as a major reservoir for *C. coli* with 89 isolates (61%)  
276 attributed with a score equal or greater than 70%. However, this proportion was greatly increased  
277 with more accurate attribution scores when using host-segregating SNPs in the attribution model.  
278 Specifically, chicken was predicted to be the source of *C. coli* infection for 138 isolates, constituting  
279 94% of the clinical samples. In comparison, two recent studies showed that sources of infection of *C.*  
280 *jejuni* are more evenly shared between chicken and cattle population in France, with approximately  
281 50% for chicken and 40% for cattle, respectively (33) (34). To draw source attribution comparisons  
282 between NA and France, additional analyses have been performed using 265 clinical isolates  
283 exclusively from the USA (Suppl Figure S2 and Suppl Table S2). The chicken source was again  
284 estimated as the main source of *C. coli* contamination in the USA as well as in France, but in a lower  
285 proportion (67.9% against 94%) followed by cattle (11.7%) and pig (20.4%). It would be interesting,  
286 in a complementary study, to compare the eating habits between these two countries.

287 In conclusion, the added resolution provided by genome-wide host-segregating markers not only  
288 improves source attribution for *C. coli* but also provides important information about the major  
289 infection reservoirs that has been missed in some previous studies (21). By combining whole  
290 genome analysis with national surveillance programs and source attribution modelling it was  
291 possible to identify the chicken reservoir as a major source of *C. coli* infection in France and abroad.  
292 These findings will support ongoing surveillance and the development of targeted interventions  
293 aimed at reducing the burden of human campylobacteriosis.



294 **MATERIAL AND METHODS**295 ***Campylobacter coli* isolate datasets**

296 A total of 450 *C. coli* isolates genomes from two major regions where Campylobacters are a leading  
297 cause of foodborne infections, North America and Europe, were selected for the determination of  
298 host-segregating markers (Dataset S1). To reduce the detection of regional-specific markers, these  
299 genomes were randomly selected from multiple countries within these two regions. That included  
300 even numbers (n=150) of chicken, cattle and pig *C. coli* genomes to avoid bias in the identification of  
301 host-specific markers. This first dataset was comprised of 151 isolates from PubMLST databases (31)  
302 and 299 from the USA National Antimicrobial Resistance Monitoring System (NARMS) project (53).  
303 PubMLST genomes comprised 34% of that first dataset and included 47%, 7% and 47% of all chicken,  
304 cattle and pig marker-determination isolates, respectively. NARMS genomes comprised 66% of the  
305 dataset, and included 53%, 93% and 53% of all chicken, cattle and pig marker-determination  
306 isolates. These datasets were entirely composed of European and North American genomes.  
307 European isolates represented 29% of the dataset, including 41%, 1% and 45% of all chicken, cattle  
308 and pig isolates respectively, while North American isolates comprised 71% of the dataset including  
309 59%, 99% and 55% of all chicken, cattle and pigs isolates. North American isolates were mostly  
310 selected from the USA (n=315). Remaining isolates (n=4) were selected from Canada. A total of 424  
311 isolates (94%) were obtained from 2005 to 2019.

312

313 A second dataset (validation dataset) comprised 300 supplementary *C. coli* isolates of known source  
314 reservoirs was used in order to test the discriminatory strength the host-segregating SNPs previously  
315 obtained (Dataset S2). This dataset comprised North American *C. coli* isolates from the NARMS  
316 project; 100 for each source. Finally, 150 French clinical isolates comprised a last set of genomes

317 (clinical dataset) and were used to attribute the putative source reservoir of clinical isolates (Dataset  
318 S3). This comprised 150 clinical isolates from French laboratories and hospitals surveillance network  
319 sampled from stools between 2015 to 2017. Clinical isolates were chosen to represent patients from  
320 diverse geographic regions in France, with a sex ratio of 1.03 and a mean age of 39.4 s.d.  $\pm$ 2.8 years  
321 old.

322

323 Clinical isolate genomes had an average genome length of 1.7 Mbp (s.d.  $\pm$  69.7 Kbp) and an average  
324 number of contigs of 43. *C. coli* marker-determination isolates were on average 1.76 Mbp (s.d.  $\pm$   
325 81.2 Kbp) in length and comprised 83 contigs; and *C. coli* validation isolates were on average 1.78  
326 Mbp (s.d.  $\pm$  74.7 Kbp) in length over 78 contigs (Suppl Figure S1). This is consistent with other  
327 published *C. coli* genomes, estimated to  $\sim$ 1.7 Mbp in length (54). Furthermore, no significant  
328 difference in *C. coli* genome sizes from different hosts has been observed: *C. coli* isolated from  
329 chickens were on average 1.78 Mbp in length (s.d.  $\pm$  106 Kbp), 1.77 Mbp (s.d.  $\pm$  61.6 Kbp) for cattle  
330 isolates and 1.77 Mbp (s.d.  $\pm$  61 Kbp) for pig isolates.

331

### 332 **DNA extraction, genome sequencing and assembly**

333 DNA from clinical isolates was extracted using the MagNA Pure 6 DNA and Viral NA SV Kit and DNA  
334 purification was performed from bacterial lysis on a MagNA Pure 96 System (Roche Applied Science,  
335 Mannheim, Germany). Quantification and purity checks (260/280 and 260/230 ratios) were  
336 determined by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA) before  
337 sequencing. Paired-end next-generation sequencing was performed on DNA samples using Illumina  
338 HiSeq 4000 technology (Integragen, Evry, France). Additionally, FastQC v0.11.8 (55) was used to run  
339 data quality tests. Genomic data was cleaned and genomes were assembled using Sickle v1.33 (56)

340 and SPAdes v3.10.1 (57), respectively. Genomes were then filtered in order to remove poor quality  
341 contigs: sequences with a length smaller than 160 nucleotides and a k-mer coverage less than 20x  
342 were removed. One isolate (2015\_0475) showed an abnormal genome size of 2.5 Mbp after  
343 filtration and was excluded from subsequent analyses.

344

#### 345 **Characterization of genomic variation**

346 *In silico*, MLST was performed for a comparative analysis with host-segregating SNPs. Profiles were  
347 obtained for all 900 isolates using 7 housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt* and *uncA*)  
348 determined for *Campylobacter* species (27). Sequence types (STs) and clonal complexes ("CC",  
349 groups of isolates with a sequence type that share four or more loci (27)) were defined using the  
350 sequence tag tool of PubMLST (58). Using this method, two clinical isolates (2016\_1990 and  
351 2017\_2288) and one validation isolate (FSIS11705596) were miss-identified as *C. coli* and were  
352 actually *C. jejuni* and removed from the dataset. The updated validation and clinical datasets were  
353 then comprised of 299 and 147 isolates, respectively. A phylogenetic tree was constructed according  
354 to all sequence types using GrapeTree (59). A second tree was built based on every host-segregating  
355 markers determined in this study, in order to make a direct comparison with the MLST tree. A multi-  
356 fasta file containing sequences from concatenated SNPs of all isolates (n=896) was created.  
357 Sequences were aligned using Muscle v3.8.1551 (60) and a Newick format tree from maximum-  
358 likelihood method was generated using Fasttree v2.1.11 (61). Microreact online platform was used  
359 to visualize the tree (62).

360

361 To identify candidate SNPs, genome-wide variant calling was primarily performed by aligning all  
362 isolates from the marker-determination dataset (n=450) to *C. coli* reference genomes. Three

363 references from each source were chosen in order to target source-specific genomic regions and  
364 capture all potential markers: OR12 strain isolated from a chicken (NZ\_CP019977.1) (63), HC2-48  
365 strain isolated from a cow (NZ\_CP013034.1) (64) and ZV1224 strain isolated from a pig  
366 (NZ\_CP017875.1) (65). The bwa v0.7.17 (66) tool developed for mapping sequences against given  
367 genomes was used here to align each isolate to OR12, HC2-48 and ZV1224 references. Alignment  
368 files were sorted using samtools v1.9 (67). Genotypes were determined with bcftools v1.9 “mpileup”  
369 variant calling tool (67), and 3 variant calling files (vcf) were generated (one for each reference). A  
370 script was written in Python (see data availability) to filter all SNP variations found in more than 2  
371 out of 3 isolates. Since a source represents 33% of the total dataset (150 isolates over 450), a  
372 proportion greater than 66% means that a same SNP variation is likely to be found in each of the 3  
373 selected sources. Therefore, this step enabled the removal of weakly discriminating polymorphisms  
374 and reduced the computational time of subsequent analyses.

375

#### 376 **Identification of host-segregating marker**

377 In order to identify host-segregating markers, source attribution tests of marker-determination  
378 isolates (of known sources) were performed using all previously selected SNPs individually to  
379 identify host-segregating markers. A matrix was constructed of all genotypes in the 450 marker-  
380 determination isolate dataset (nucleotides were translated into numbers: “1” for “A”, “2” for “T”,  
381 etc.). Source attribution tests were performed in triplicate for each SNP using STRUCTURE (68), with  
382 the no admixture model, 3 putative populations ( $K = 3$ ), 10,000 iterations, and a burn-in period of  
383 10,000 iterations. For each STRUCTURE test, 60 different random isolates (20 from each population)  
384 were set to “unknown source” (POPFLAG = 0) in order to estimate the probability of correct self-  
385 attribution, and then to evaluate the SNP host-segregating strength. Each SNP with 70% or greater

386 of total correct self-attributions for at least one source was selected; a minimum of 66% (here  
387 rounded up to 70%) of source attribution rate indicates that a variant is discriminating between at  
388 least 1 out of 3 sources. Additionally, genomic sequences containing the selected SNPs were  
389 extracted from the corresponding reference (OR12, HC2-48 or ZV1224) and annotated using blast-x  
390 online tool (69).

391

### 392 **Validation of the discriminatory power of host-segregating markers**

393 To validate the capability of the selected SNPs to discriminate isolates from different populations,  
394 STRUCTURE tests were run again using the marker-determination dataset and different sets of  
395 markers: SNPs contained in the same CDS, all SNPs determined from OR12, HC2-48 and ZV1224  
396 alignments and all SNPs from all alignments. One hundred tests were then performed using each set  
397 of SNPs and 60 random isolates per test for self-attribution (POPFLAG = 0) ("no admixture model", K  
398 = 3, 10,000 iterations and a burn-in period of 10,000 iterations). Additionally, source attribution of  
399 299 validation isolates of known source reservoirs, which were not used for SNP determination, was  
400 performed. Specifically, each SNP was obtained using samtools mpileup option. STRUCTURE was run  
401 10 times using marker-determination isolates as training dataset (n=450) and validation dataset as  
402 unknown source isolates (POPFLAG = 0). STRUCTURE model parameters remained unchanged. Each  
403 validation isolate was attributed to its source based on the average of attribution rate of all 10 tests.  
404 An isolate was considered correctly source re-attributed with a STRUCTURE score greater than 70%.  
405 In each case the same method was performed simultaneously with MLST alleles to compare the  
406 discriminating strength of both type of markers (SNP or allele).

407

408

409 **Source attribution of clinical isolates**

410 Similar to validation analysis, source attribution of *C. coli* clinical isolates was performed using  
411 determined host-segregating markers thus in order to identify the main source of infection in  
412 France. For each SNP (n=259), every genotype was extracted from all clinical isolates using samtools  
413 mpileup option. STRUCTURE was run 10 times using marker-determination isolates as training  
414 dataset (n=450) and clinical dataset (n=147) as unknown source isolates (POPFLAG = 0) (K = 3,  
415 10,000 iterations and a burn-in period of 10,000 iterations). Each clinical isolate was attributed to a  
416 source based on the average of attribution rate of all 10 tests. Source attribution of clinical isolates  
417 was performed simultaneously with MLST alleles to compare proportions of each source between  
418 both type of markers (SNP or allele).

419 **DATA AVAILABILITY**

420 All 900 *C. coli* genomes are available using IDs listed in Dataset S1, S2 and S3: BioSample and  
421 PubMLST IDs for NCBI and PubMLST databases respectively.

422

423 Personal VCF filter Python script available on GitHub: QuentinJehanne. (2020, April 8).

424 QuentinJehanne/ccoli\_2020: v1 of a personal VCF filter (Version v1.0.0). Zenodo.

425 <http://doi.org/10.5281/zenodo.3744758>.

426

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433

434 **AUTHOR CONTRIBUTIONS STATEMENT**

435 Conceptualization, Quentin Jehanne and Philippe Lehours; Funding acquisition, Philippe Lehours;  
436 Investigation, Quentin Jehanne, Ben Pascoe and Samuel K Sheppard; Project administration, Quentin  
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438 Alice Buissonnière, Evangelos Mourkas, Ben Pascoe and Samuel K Sheppard; Supervision, Philippe  
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440 Lehours; Visualization, Quentin Jehanne, Ben Pascoe, Samuel K Sheppard and Philippe Lehours;  
441 Writing – original draft, Quentin Jehanne and Philippe Lehours; Writing – review & editing, Quentin

- 442 Jehanne, Samuel K Sheppard, Ben Pascoe, Francis Mégraud, Emilie Bessède and Philippe Lehours. All  
443 authors have read and agreed to the published version of the manuscript.



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655 **Tables**

656

657 **Table 1. Variant calling comparison between 3 references of *C. coli***

658

659 **Table 2. Rates of correct self-attributions of marker-determination isolates using 5 different set of**  
660 **markers**

661

662 **Table 3. Source attribution scores of clinical isolates**

663 **Figure Legends**

664

665 **Figure 1: Phylogenic tree based on MLST analysis**

666 The minimum spanning tree was generated using GrapeTree from the sequence types of all 896 *C.*  
667 *coli* isolates, based on 7 MLST genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt* and *uncA*) extracted using  
668 PubMLST platform. Orange color represents isolates isolated from chickens, green color from cattle,  
669 magenta color from pigs and red color are for clinical isolates. Circle sizes are proportional to the  
670 number of isolates and the scale bar represents a genetic distance of 1.

671

672 **Figure 2: Phylogenic tree built from concatenated selected SNPs**

673 Tree designed using maximum-likelihood phylogeny between 896 isolate sequences built from the  
674 concatenation of all genotypes of the selected SNPs (n=259). Orange nodes are the chicken  
675 population isolates, green nodes for cattle isolates, pink nodes for pig isolates and red nodes for  
676 clinical isolates. Orange circle shows an estimation of the chicken cluster, green circle for the cattle  
677 cluster and pink circle for the pig cluster and the scale bar represents a genetic distance of 0.24.  
678 Clinical isolates are mostly located within the chicken cluster, which is consistent with the  
679 probabilistic attribution model.

680

681 **Figure 3: Host-segregating rate of all variants obtained from the alignment of 450 marker-**  
682 **determination isolates against 3 references**

683 Source attribution rates (y axes) were obtained testing 26,131, 24,395 and 20,827 SNPs from OR12  
684 (a), HC2-48 (b) and ZV1224 (c) references, respectively, and are shown here according to their  
685 genome position (left, x axis) and variant proportions (right, x axis). STRUCTURE software was run 3

686 times for each SNP (average attribution rates are shown here), using 390 *C. coli* randomly selected  
687 isolates as training dataset and 60 randomly selected isolates as test dataset. Orange color  
688 represents attribution rates and number of SNPs for chicken source, green color for cattle source  
689 and magenta color for pig source. A total of 259 SNPs showed attribution rates greater than 70%  
690 (red line) for one or more sources and were carried forward for further analyses: 43, 183 and 33  
691 SNPs from chicken, cattle and pig references, respectively. Scores fluctuated between 30% and 40%  
692 and highest attribution rates for each host reservoir were found in the corresponding source  
693 reference. However, OR12 reference showed two distinct regions of the genome: one part  
694 containing variants discriminating the chicken source and another part the pig source. Two low  
695 variable regions (blanks), where no SNP from the variant calling step were selected, are also visible.  
696

697 **Figure 4: Correct re-attribution proportions of 299 validation isolates using determined SNPs and**  
698 **MLST genes**

699 Source attribution strength of selected SNPs (**a**) and MLST genes (**b**) estimated using STRUCTURE  
700 software. A total of 299 isolates were tested (from the validation dataset) using marker-  
701 determination isolates for training (n=450). Source attributions were performed 10 times using all  
702 selected SNPs (n=259) and MLST genes (n=7). Gray bars represent rate of correct source attribution  
703 for chicken population isolates, black bars for cattle isolates and white bars for pig isolates. An  
704 isolate was considered correctly source re-attributed with a STRUCTURE score greater than 70%.  
705

706 **Figure 5: Population proportions of clinical isolates from source attribution**

707 Source attribution of clinical dataset using selected SNPs (**a**, n=259) and MLST genes (**b**, n=7). Clinical  
708 isolates (n=147) are represented on x axis and their attribution probabilities on y axis in orange for

709 chicken source, green for cattle source and pink for pig source. The poultry reservoir was estimated  
710 as the main source of *C. coli* contamination in France with 138 isolates (94%) attributed using host-  
711 segregating SNPs and 89 isolates (61%) using MLST (isolates selected with source probabilities  
712 greater than 70%).

713 **Supplementary Tables and Figures**

714

715 **Dataset S1. Marker-determination dataset isolates**

716

717 **Dataset S2. Validation dataset isolates**

718

719 **Dataset S3. Clinical dataset isolates**

720

721 **Suppl Figure S1. WGS data from all 900 *C. coli* isolates.**

722

723 **Suppl Table S1. List of all determined proteins with their corresponding number of SNPs.**

724

725 **Suppl Figure S2. Population proportions from source attribution of 265 *C. coli* clinical isolates from**  
726 **the USA.**

727

728 **Suppl Table S2. List of all *C. coli* isolates from the USA selected for source attribution.**

729

730

**Table 1. Variant calling comparison between 3 references of *Campylobacter coli***

Reference	Variant calling raw <sup>1</sup>	Filtration <sup>2</sup>	Selected SNPs <sup>3</sup>
OR12 (chicken)	283,320	26,131	43
HC2-48 (cattle)	202,111	24,395	183
ZV1224 (pig)	242,574	20,827	33

<sup>1</sup>Number of SNPs determined after aligning all isolates from marker-determination (n=450) dataset to 3 different references of *C. coli* : OR12 isolated from chicken, HC2-48 from cattle and ZV1224 from pig.

<sup>2</sup>Number of SNPs after the filtration of genotypes which represent more than two third of all isolates.

<sup>3</sup>Selected SNPs with 70% or greater of total correct self-attributions.



**Table 2. Rates of correct self-attributions of marker-determination isolates using 5 different set of markers**

Set of markers	Chicken isolates self-attributions (n = 150)		Cattle isolates self-attributions (n = 150)		Pig isolates self-attributions (n = 150)	
	Rate of correct attribution (%)	Std deviation (%)	Rate of correct attribution (%)	Std deviation (%)	Rate of correct attribution (%)	Std deviation (%)
43 SNPs (OR12)	88.4	± 6.24	63.8	± 9.22	96.2	± 4.09
183 SNPs (HC2-48)	91.1	± 5.74	75.0	± 9.69	42.5	± 18.74
33 SNPs (ZV1224)	75.0	± 13.9	19.7	± 10.08	94.7	± 5.23
<b>259 SNPs (all)</b>	<b>92.0</b>	<b>± 5.86</b>	<b>77.0</b>	<b>± 8.65</b>	<b>95.3</b>	<b>± 4.4</b>
<b>7 genes (MLST)</b>	<b>73.6</b>	<b>± 9.06</b>	<b>76.8</b>	<b>± 9.42</b>	<b>74.4</b>	<b>± 9.50</b>

Discriminating strength of selected SNPs and MLST genes were estimated using marker-determination isolates. From 450 initial isolates, random selections of 390 and 60 isolates were used for training and self-attribution (sources set to "unknown"), respectively. Self-attributions were performed 100 times using selected SNPs from chicken alignment (n=43), from cattle alignment (n=183), from pig alignment (n=33), from all alignments (n=259) and 50 times using MLST genes (n=7). Since multiple tests were performed for each set of markers using 60 randomly selected isolates, standard deviations were calculated.

**Table 3. Source attribution scores of clinical isolates**

Set of markers	Attribution to chicken source	Attribution to cattle source	Attribution to pig source	Inconclusive attributions
	% of clinical isolates (Average score %)	% of clinical isolates (Average score %)	% of clinical isolates (Average score %)	% of clinical isolates (Average score %)
259 SNPs	93.88 (100.0)	0.0 (0.0)	6.12 (100.0)	0.0 (0.0)
7 MLST genes	60.54 (88.35)	8.84 (86.91)	4.08 (83.22)	26.53 (50.59)

Data for source attribution of clinical isolates dataset using selected SNPs (n=259) and MLST genes (n=7). “% of clinical isolates” show the distribution of estimated sources among clinical isolates with “Average score” as their average of individual attribution rate. Using determined SNPs, source attribution rates for clinical isolates were constant whereas using MLST genes, source attribution showed variable results.









