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## Diversity of *Escherichia coli* strains isolated from day-old broiler chicks, their environment and colibacillosis lesions in 80 flocks in France

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1 Diversity of *Escherichia coli* strains isolated from day-old broiler chicks, their environment  
2 and colibacillosis lesions in 80 flocks in France

3

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**24 Abstract**

25 Avian colibacillosis is the most common bacterial disease affecting broilers. To better  
26 evaluate the diversity and the origin of the causative *Escherichia coli* strains infecting birds,  
27 we conducted a study on 80 broiler flocks. Just before the arrival of chicks on the farm,  
28 samples were collected in the farm environment (walls, feeders, air inlets, etc.) and, upon  
29 delivery, day-old chicks (DOCs) and the transport boxes were also sampled. Isolates were  
30 obtained from these samples, and from organs of chickens exhibiting typical colibacillosis  
31 symptoms. The isolates were characterized using high-throughput qPCR to detect a range of  
32 genetic markers (phylogroups, main serogroups virulence markers, etc.). A total of 967  
33 isolates were studied, including 203 from 28 colibacillosis episodes, 484 from DOCs, 162  
34 from transport boxes and 118 from the farm environment. These isolates yielded 416 different  
35 genetic profiles, of which 267 were detected in single isolates, and the others were observed  
36 in up to 44 isolates from nine farms. The distributions of isolates across phylogroups and the  
37 main serogroups varied with the origin of isolation. The isolates obtained from colibacillosis  
38 cases either shared a single genetic profile or were different. In a few cases, we observed the  
39 same profile for isolates obtained from DOCs and colibacillosis lesions in the same flock or  
40 different flocks. However, some flocks receiving DOCs contaminated with isolates bearing  
41 the genetic profile of colibacillosis cases identified in other flocks remained healthy. This  
42 study highlights the huge diversity of avian pathogenic and non pathogenic *E. coli* strains.

43

44

**45 Keywords**

46 Broilers - Colibacillosis - Strain - Diversity

47

48

## 49 **Introduction**

50 Avian colibacillosis is the most common infectious bacterial disease in poultry (Nolan et al.,  
51 2020; Souillard et al., 2019), leading to significant economic losses. It can affect various  
52 poultry species (chickens, turkeys, ducks, etc.), different production breeds (broilers, layers,  
53 breeders) and production types (conventional, organic). Clinical signs may be diverse, but  
54 colibacillosis in poultry is most typically a localized or a systemic extra-intestinal disease. In  
55 chicks, the systemic form of colibacillosis is called colisepticemia and results in early  
56 mortality. Typical lesions include polyserositis affecting air sacs, the pericardium, pleura and  
57 the peritoneum. Later on, other syndromes may be observed, including respiratory signs or  
58 lameness. *Escherichia coli* can be isolated from the lesions, but this bacterium is also present  
59 in the farm environment, and is a normal component of poultry intestinal microbiota, with  
60 titres of  $10^6$  colony forming units per gram of faecal material. Several studies have attempted  
61 to identify virulence markers to differentiate the pathogenic strains involved in clinical cases,  
62 called avian pathogenic *E. coli* (APEC), from the non-virulent strains. Lists of these virulence  
63 markers or virulence-associated genes (VAGs) and diagnostic schemes have been proposed  
64 (Johnson et al., 2008; Schouler et al., 2012). However, it is still not possible to diagnose all  
65 APEC isolates without ambiguity (Guabiraba and Schouler, 2015), because no specific gene  
66 can be used as a hallmark to distinguish virulent strains from non-pathogenic strains (Nolan et  
67 al., 2020). Moreover, although highly virulent APEC strains may exist, colibacillosis is  
68 sometimes considered as a secondary disease caused by opportunistic bacteria when host  
69 defences are impaired, due to other infections, immunosuppression or poor production  
70 conditions.

71 The origin of the APEC strains affecting a flock is most often unknown. Strains with VAGs  
72 are present in the intestinal microbiota of healthy birds, with one report demonstrating that  
73 24% of the intestinal *E. coli* isolated from day-old chicks carried more than five VAGs

74 (Kemmett et al., 2013). Strains with VAGs can also be detected in the farm environment  
75 (litter, dust, housing material, feed, wild birds, rodent droppings, insects, well water, etc.  
76 (Nolan et al., 2020)). In a few cases, the strain colonizing or infecting the chicks originates  
77 from the breeder (parental) flock (Pasquali et al., 2015; Petersen et al., 2006). A breeder or  
78 hatchery origin may be suspected when very similar isolates are isolated from colibacillosis  
79 lesions of birds grown in different flocks, but originating from the same breeder flock or the  
80 same hatchery. For example, one study (Petersen et al., 2006) identified the same  
81 enrofloxacin-resistant clone in dead broilers from five flocks and in cloacal swabs from one  
82 parental flock, eggs and hatchery fluff, thus demonstrating vertical transmission. There are  
83 various methods aiming to compare isolates from different farms, including phenotypic  
84 methods such as serotyping, multilocus enzyme electrophoresis, outer membrane protein  
85 profiles and antimicrobial sensitivity testing (Giovanardi et al., 2005; Turret and Denamur,  
86 2016), as well as genotypic methods such as pulsed field gel electrophoresis (PFGE), random  
87 amplified polymorphic DNA (RAPD), multilocus sequence typing (MLST), PCR-based  
88 phylogrouping and virulence genotyping (Dissanayake et al., 2014; Pasquali et al., 2015). The  
89 most recent method, whole-genome sequencing, can even compare chromosomal or plasmid  
90 contents of isolates (Olsen et al., 2012; Poulsen et al., 2018). However, this powerful method  
91 is time consuming and expensive to apply to large numbers of isolates. Therefore, high-  
92 throughput PCR methods appear to be more convenient and less costly for diagnosis and large  
93 epidemiological investigations (Baranzoni et al., 2016).

94 The aim of this study was to better determine the origin of colibacillosis strains. Thus, we  
95 conducted a longitudinal epidemiological study on 80 commercial broiler flocks located in  
96 western France, the main French region for poultry production. We collected *E. coli* strains  
97 from chicks, their environment and from lesions in colibacillosis cases. The *E. coli* strains  
98 were characterized using high-throughput PCR to detect a panel of genetic markers. Here, we

99 describe the diversity and traceability within and between flocks of more than 900 *E. coli*  
100 isolates.

101

## 102 **Materials and methods**

### 103 Farms and flocks

104 Eighty standard broiler flocks (breeds: Ross RJE, Ross 308 or Cobb 500), vaccinated against  
105 infectious bronchitis and Gumboro disease and bred in one of four major poultry production  
106 companies, were included in the study. The flocks were grown in the main production areas in  
107 western France between May 2017 and February 2019. Each flock was visited twice, first on  
108 the first day of chick delivery from the hatchery, and at three weeks of age.

109 On the first day, one hour before the chicks arrived, five swab samples were collected in the  
110 farm's broiler house (walls, feeders, troughs, air inlets, and litter), and the water was sampled.  
111 This whole set of samples from the farm environment is hereafter called "farm". Upon chick  
112 arrival, 10 day-old chicks (DOCs) were sampled and one swab of 20 delivery boxes was  
113 collected directly in the lorry; this latter sample is hereafter called "transport".

114 For the 80 initially included flocks, it was agreed that, suspicion of early colibacillosis was  
115 defined as a flock of up to 10 days of age with a daily mortality rate higher than 0.3% and  
116 suspect clinical signs or lesions, whereas late colibacillosis was defined as birds aged more  
117 than 10 days with a daily mortality higher than 0.1% on two consecutive days and suspect  
118 clinical signs or lesions. In the case of suspicion, the farmer brought 10 chicks (early  
119 colibacillosis) or 6 chickens (late colibacillosis) to the laboratory.

### 120 *E. coli* isolation

121 In the laboratory, harmonized standard protocols were used to isolate *E. coli*, farm and  
122 transport samples and from the yolk and the liver of the 10 DOCs. For the early colibacillosis  
123 cases, 10 chicks were necropsied, and if there were typical colibacillosis lesions on at least

124 four chicks, lesions from the air sacs, liver, spleen, joint or heart tissues were inoculated on  
125 lactose media. For late colibacillosis cases, six chickens were necropsied and lesions of the  
126 liver, spleen, heart, joints, or air sacs from at least four affected chickens were inoculated on  
127 lactose media. The *E. coli* strains were then identified according to standard methods  
128 (MALDI-TOF or biochemical characteristics), and DNA extracts were prepared from  
129 colonies, using InstaGene™ (Bio-Rad, Marnes La Coquette, France). The maximum number  
130 of isolates collected at each visit is given in Table 1.

### 131 High-throughput PCR

132 A high-throughput microfluidic real-time PCR (qPCR) system was used to screen genetic  
133 markers related to 23 serogroups (O1 (2 variants), O2 (3 variants), O6, O8, O11, O18, O23,  
134 O25, O35, O45(S88), O78, O88, O153, H4, H7, H8, H21, H25, K1, K5), five phylogroup  
135 markers (Clermont et al., 2013; Clermont et al., 2019) and 66 virulence factors  
136 (Supplementary Table 1), in addition to the *cdgR* gene (species marker).

137 qPCR amplifications were performed using the BioMark™ real-time PCR system (Fluidigm,  
138 South San Francisco, CA, USA) and 96.96 dynamic arrays (Fluidigm), using 6-  
139 carboxyfluorescein (6-FAM)- and Black Hole Quencher® (BHQ1)-labelled TaqMan probes  
140 according to the manufacturer's instructions.

141 The genetic profile of the isolates was obtained by concatenating the qPCR-detected genes,  
142 and the Simpson's diversity indexes (Simpson, 1949) of the isolates from farm and transport  
143 swabs, DOCs, early colibacillosis cases and late colibacillosis cases were calculated.

144 Unbiased hierarchical clustering with heatmap generation was generated using the R package  
145 ComplexHeatmap (version 2.2.0) (Gu et al., 2016). The distance matrix was estimated using  
146 the Euclidean method and the ward.D method was used to cluster the distance matrix.

### 147 Antimicrobial susceptibility testing

148 Susceptibility to 15 antimicrobials was determined according to the French standard NF U 47-  
149 107 for sub-sample of 73 isolates, based on similar genetic profiles of isolates obtained from  
150 different animals or flocks.

#### 151 Statistical methods

152 Statistical methods

153 The distribution of positive and negative results was compared using a chi-squared or Fisher's  
154 exact test ( $n \leq 5$ ). It allows to study the relationships between categorical variables taken in  
155 pairs. For all tests, values of  $p < 0.05$  were considered statistically significant differences.

156 To have an overall vision of the relationships between the categorical variables under study  
157 (origin, serogroup and phylogroup), a Multiple Correspondence Analysis (Greenacre, 1984)  
158 was applied.

159

## 160 **Results**

### 161 Number of colibacillosis and number of strains

162 Among the 80 flocks included, 20 flocks suffered from early colibacillosis only, 6 from late  
163 colibacillosis only, and 5 flocks encountered both. Thus, overall, 31 flocks had at least one  
164 colibacillosis episode. A total of 967 isolates were available for study (Table 1).

### 165 Diversity of strains

166 All 967 isolates carried *cdgR*, confirming the *E. coli* species identification. The *ompA* and the  
167 *papG* allele I genes were found in all isolates, but neither the *focG*, LT, STa, STb, *stx1* nor the  
168 *stx2* genes were found in any of the isolates. These genes were thus excluded from the genetic  
169 profiling study.

### 170 Serogroups

171 A total of 421 isolates (43.5%) belonged to 12 different serogroups (Figure 1). Isolates for  
172 which the serogroup could not be determined represented 45.7% (n=138), 24.6% (n=65),



173 54.1% (n=484), 68.5% (n=162) and 79.7% (n=118) of farm and transport samples, DOCs,  
174 early colibacillosis cases and late colibacillosis cases (Pearson's chi-squared test,  $p < 0.001$ ),  
175 respectively. Thus the number of determined and undetermined serogroups varied according  
176 to the origin of the isolates, except for the farm and transport samples, DOCs and early  
177 colibacillosis cases.

178 The distribution of the serogroups varied with the source of isolation (Table 2). The numbers  
179 of O2 and non-O2 serogroups varied between isolate origins, except between DOCs (18% of  
180 O2 isolates) and early colibacillosis cases (22% of O2 isolates), between farm samples (3% of  
181 O2 isolates) and late colibacillosis cases (0% of O2 isolates) and between farm (3% of O2  
182 isolates) and transport (9% of O2 isolates) samples. The percentage of O8 isolates was  
183 significantly higher in DOCs (14%) than in colibacillosis cases (3% for early ( $p = 0.0002$ ) and  
184 0% for late colibacillosis ( $p = 0.0003$ )) and more O8 isolates were detected in transport  
185 samples (11%) than in early colibacillosis cases (3%) ( $p = 0.007$ ). Regarding the O25  
186 serogroup, MCA showed that late colibacillosis isolates belonged significantly more often to  
187 the O25 serogroup (22% of O25 isolates, originating from three flocks) than the isolates from  
188 other origins (maximum of 2% of O25 isolates,  $p < 10^{-5}$ ) (Figure 2). In all, 38.5% of late  
189 colibacillosis isolates belonged to the O78 serogroup, compared with less than 0.2% in the  
190 other isolate groups ( $p < 2 \times 10^{-13}$ ) (Figure 2). The distributions of O88 and non-O88 were not  
191 significantly different between early and late colibacillosis isolates (15% of O88 isolates for  
192 each), and there were significantly more O88 isolates in colibacillosis cases than in farm  
193 isolates ( $p < 2.10^{-5}$ ), transport isolates ( $p < 0.0003$ ) and DOCs ( $p < 2 \times 10^{-5}$ ) (less than 3% for  
194 each).

### 195 Phylogroups

196 The phylogroups of isolates according to isolate origin are given in Figure 3.

197 Significantly fewer late colibacillosis isolates belonged to phylogroup A (0% compared with  
198 more than 8% for isolates from other origins,  $p < 0.03$ ) (Table 2). Isolates from early or late  
199 colibacillosis cases belonged significantly less frequently to phylogroup B1 compared with  
200 isolates from other origins (22% and 23%, respectively, compared to a minimum of 44% for  
201 other groups of isolates,  $p < 4 \times 10^{-5}$  for early colibacillosis,  $p < 0.003$  for late colibacillosis).  
202 There were significantly more isolates belonging to the B2 phylogroup among those obtained  
203 from early colibacillosis (33%) than those from DOCs (21%) ( $p = 0.003$ ), transport samples  
204 (14%) ( $p = 9 \times 10^{-5}$ ) or farm samples (2%) ( $p = 3 \times 10^{-12}$ ), and significantly fewer isolates from  
205 farm samples belonged to this phylogroup compared with all other origins ( $p < 0.0002$ ). Late  
206 colibacillosis isolates belonged less frequently to phylogroup D/E (1.5%) (Figure 2) than  
207 isolates from DOCs, transport and farm swabs (respectively 8.5%, 11% and 15%, ( $p < 0.05$ )).  
208 Significantly more isolates from colibacillosis cases fell in phylogroup F/G (25% and 35% for  
209 early and late colibacillosis respectively,  $p < 0.01$  and  $p < 0.0002$  respectively) than isolates of  
210 other origins; furthermore, only 1% of farm isolates belonged to this phylogroup, compared  
211 with percentages of higher than 8% for the other origins ( $p < 0.003$ ).  
212 Table 2 summarizes the significant and non-significant differences of the serogroup and  
213 phylogroup distributions between DOC and early and late colibacillosis isolates. MCA also  
214 revealed associations between serogroup O1 and phylogroup B2 ( $p = 5 \times 10^{-9}$ ), and serogroup  
215 O2 and phylogroup B2 ( $p = 8 \times 10^{-7}$ ).

#### 216 Diversity of genetic profiles

217 When combining the results obtained for the different genetic markers for each isolate, 416  
218 different profiles were detected (Figure 4). Among these, 267 were unique profiles  
219 (singletons), observed in one single isolate, 99 were observed two to four times (55 twice, 23  
220 three times, 21 four times) and 50 profiles were observed more than four times. The most

221 frequent profiles were Pf1 (44 isolates observed on nine farms), Pf2 (identified 19 times on  
222 one farm) and profiles Pf3 to Pf14, which were detected more than 10 times each.

223 The values of Simpson's diversity index for early colibacillosis, late colibacillosis, DOCs,  
224 transport and farm samples were respectively 0.980, 0.980, 0.978, 0.978 and 0.974.

225 Table 3 gives the origin of the isolates for the 10 most frequent profiles. Some of these  
226 profiles were observed in only one type of sample from a single flock, e.g. Pf6, Pf 9 and Pf10  
227 were detected only in isolates from, respectively, the farm environment for the L4 flock, late  
228 colibacillosis cases for the P33 flock, and DOCs for the P42 flock. Some profiles were  
229 detected in different types of samples, but never or rarely in colibacillosis cases. For instance,  
230 Pf1 was detected in DOCs (39 isolates from seven farms) and in transport samples (four  
231 isolates from three farms), but only in one colibacillosis isolate from another farm. Pf2 was  
232 detected in 19 isolates from flock L23, either in DOCs or transport samples, but was never  
233 detected in colibacillosis cases. Pf4 was detected in 15 isolates from DOCs, farm or transport  
234 samples, but never in colibacillosis cases. Interestingly, Pf3 was detected in only two flocks:  
235 in the P5 and P8 flocks, it was identified in DOCs and in early colibacillosis cases, however in  
236 flock P5, Pf3 was the unique profile of the six colibacillosis isolates studied, whereas in P8,  
237 the eight colibacillosis isolates showed five different profiles. Pf5, Pf7 and Pf8 were identified  
238 in isolates from early colibacillosis cases, DOCs and transport swabs. Interestingly, Pf7 was  
239 detected in both types of colibacillosis cases, DOCs and transport swabs from the L9, L14,  
240 P11 and P25 flocks. The Pf5 and Pf8 isolates were not isolated from colibacillosis and non-  
241 colibacillosis samples from the same flocks.

242 The 21 cases of early colibacillosis, for which 138 isolates were available, yielded 65 profiles,  
243 with 34 singletons. One to 12 (mean 6.6) isolates per case were studied. For each case, one to  
244 eight profiles were detected, with a mean of 3.5 profiles per case. From each chick, we  
245 obtained one or two profiles. Most early colibacillosis profiles were detected in a single flock,

246 but six profiles were detected in two flocks, and one profile (Pf5) was present in three flocks  
247 (L9, L17 and L24). Each early colibacillosis profile was obtained from one to seven chicks.  
248 For the seven cases of late colibacillosis for which 65 isolates were available, 4 to 14 (mean  
249 9.3) isolates per case were analysed, yielding 19 different profiles. For each case, one to four  
250 profiles were detected, with a mean of 2.9 profiles per case. All 19, except Pf13, were  
251 detected in late colibacillosis isolates from a single flock and obtained from one to four  
252 different birds of this flock. Pf13 was detected from nine diseased chickens from two flocks  
253 (L1 and L3).

254 For DOCs, 209 profiles, 114 being unique, were recorded in the 484 isolates. Each profile was  
255 detected in 1 to 39 DOC isolates, 1 to 28 different DOCs and DOCs from one to seven flocks.  
256 We tracked the most frequent colibacillosis profiles. For early colibacillosis cases, the Pf16  
257 profile was identified in four and five colibacillosis isolates from respectively flocks L17 and  
258 L32, but was never detected in DOCs, farm or transport samples. Similarly, the Pf18 profile  
259 was obtained from only eight colibacillosis isolates from the L28 flock. As mentioned above,  
260 the Pf3 profile was observed in the P5 and P8 flocks' colibacillosis cases, but this profile was  
261 also detected in DOCs from these two flocks. The Pf5 profile was present in colibacillosis of  
262 the L17 (three isolates), L24 (two isolates) and L9 (one isolate) flocks, and in one DOC in the  
263 P19 flock, and in seven isolates from farm swabs for the L18, P2 and P15 flocks.

264 Interestingly, flocks P19 and P2 did not report any colibacillosis cases, whereas the L18 and  
265 P15 flocks showed an early colibacillosis case with isolates displaying other profiles.

266 Eighteen of the 19 late colibacillosis profiles were observed in colibacillosis isolates only.

267 The Pf13 profile was recorded in late colibacillosis isolates from two different flocks (L1 and  
268 L3), Pf22 was identified in early and late colibacillosis isolates from the P44 flock, and Pf23  
269 and Pf41 were detected in early colibacillosis (L5 or L18) and late colibacillosis (P21)  
270 isolates.

271 Hierarchical clustering of the isolates according to 58 VAGs (excluding serogroup and  
272 phylogroup information) as part of the heatmap analysis identified two main clusters (Figure  
273 4). Interestingly, these clusters could be clearly distinguished by their phylogroup. One cluster  
274 (on the right-hand side of the heatmap) contained all of the isolates from phylogroups B2,  
275 F/G, E/C11/U and some isolates from phylogroup D/E, whereas the other cluster grouped all  
276 of the isolates from phylogroups A, B1, A/C/C11, and C11/C12/U and the remaining isolates  
277 from phylogroup D/E. These two clusters appeared to be mainly differentiated by the  
278 presence/absence pattern of the cluster of VAGs at the bottom of the heatmap, i.e. *tsh*, *vat*,  
279 *fyuA*, *YqiC*, *frz<sub>orf4</sub>*, *tkl1*, *aec4*, *csgA2* and *ibeA*.

280 One sub-cluster of the A/B1 cluster (located on the right-hand side of the cluster on the  
281 heatmap) clearly differed by the absence of the VAGs located at the top of the heatmap, i.e.  
282 the APEC ColV plasmid-associated VAGs. Interestingly, none of the isolates from this sub-  
283 cluster lacking the APEC ColV plasmid were isolated from a colibacillosis case.  
284 Other than this sub-cluster, no other clear associations between VAG and isolate origin were  
285 detected.

#### 286 Antimicrobial susceptibility profiles

287 The susceptibility of isolates sharing the same genetic profiles but obtained from different  
288 animals or different flocks was analysed. Among the 12 Pf9 isolates from the late  
289 colibacillosis case of flock P33, three isolates obtained from chicks 2 and 3 were resistant to  
290 amoxicillin and tetracycline, whereas nine obtained from chicks 1 to 4 were resistant to  
291 amoxicillin, tetracycline and trimethoprim-sulfamethoxazole. In some cases, the same  
292 resistance profile was observed for isolates sharing identical genetic profiles, obtained from  
293 different chicks from different flocks: all early colibacillosis Pf45 isolates from chicks 7 and 8  
294 from flock L1 and from chicks 1 and 2 from flock L32 were pan-susceptible. This was also  
295 the case for the early-colibacillosis tetracycline-resistant Pf90 isolates obtained from flocks

296 L14 (two isolates) and L27 (one isolate). But, for Pf7 isolates obtained from different flocks,  
297 the two isolates from flock P11 were susceptible to all tested antibiotics, the four from P25  
298 were resistant to nalidixic acid only, and the six isolates from L9 and L17 were resistant to  
299 third-generation cephalosporins, tetracycline and gentamicin.

## 300 **Discussion**

301 This epidemiological study was a unique opportunity to obtain and analyse many *E. coli*  
302 isolates, including isolates from DOCs, the farm environment and colibacillosis lesions, from  
303 different flocks in a large French region during a 20-month period.

304 Using numerous genetic markers included in the high-throughput qPCR study, our isolates  
305 showed high diversity. The Simpson diversity index values were similar between origins. The  
306 huge diversity of the *E. coli* genome has already been highlighted, giving estimates of the  
307 pangenome of around 10 times the size of the core genome, with as many genes in the *E. coli*  
308 species as in the human species (Tourret and Denamur, 2016).

309 Some of the main characteristics of the colibacillosis isolates along with the other origins was  
310 the high proportion of isolates belonging to the F/G phylogroup, and the low proportion to the  
311 B1 phylogroup. Phylogroup F/G can be detected using the “revisited” PCR protocol described  
312 in 2013 (Clermont et al., 2013), but not the initial method (Clermont et al., 2000). This  
313 phylogroup is composed of strains previously assigned to the D phylogroup, and is, according  
314 to the above-cited studies, a sister group of phylogroup B2. In a study of 272 APEC from the  
315 UK, Italy and Germany, the most prevalent phylogroup was B2 (47%), with differences  
316 between countries (Cordoni et al., 2016). The B2 phylogroup also predominated among the  
317 APEC in a study of salpingitis and peritonitis in 68 Danish broiler breeders (Pires-dos-Santos  
318 et al., 2013). Our results suggest that the B2 phylogroup is associated with poultry affected  
319 with early colibacillosis and that this phylogroup has a low survival rate in a farm  
320 environment. However, this predominance of B2 among APEC has not been observed in other

321 studies, which report APEC clusters in the A, B1 and D phylogroups, with fewer than 20% of  
322 B2 isolates (Pasquali et al., 2015; Tourret and Denamur, 2016). Nonetheless, because the  
323 colibacillosis isolates were not tested in an experimental animal model, their pathogenic  
324 potential cannot be determined. Conversely, the A phylogroup is regularly described as the  
325 commonest phylogroup in commensal *E. coli* from poultry. In one study on broilers and their  
326 breeder flocks in Italy, associations were found between the organ of origin of the isolate and  
327 the phylogroups: the B1 phylogroup was associated with the gut, reflecting the predominance  
328 of B1 in our farm and transport samples, which are probably mainly contaminated with faecal  
329 material; the A and D phylogroups were associated with dust and the respiratory tract, and B2  
330 with internal organs (liver, marrow and spleen) (Pasquali et al., 2015). This Italian study also  
331 described associations with poultry age, with the B1 phylogroup corresponding to DOCs, as  
332 in our study, and the A and D phylogroups corresponding to older broilers, contrary to our  
333 low percentage of phylogroup A in late colibacillosis, but similar to our relatively high  
334 proportion of isolates belonging to phylogroups D or F/G in late colibacillosis.

335 Among the genes mostly associated with the cluster of B2 and F/G phylogroup isolates, *vat*,  
336 *fyuA*, *tkt1* and *ibeA* have previously been shown to be associated with phylogroup B2 (Li et  
337 al., 2012; Nojoomi and Ghasemian, 2019; Spurbeck et al., 2012). The association between  
338 APEC of the O1 and O2 serogroups and the B2 phylogroup was previously observed by  
339 Moulin-Schouleur et al. (2007), who reported that 28 of the 30 APEC strains of serogroups  
340 O1, O2, and O18 were classified by MLST into the same subcluster (B2-1) of phylogenetic  
341 group B2.

342 Regarding serogroups, although we included primers for 12 O-, 5 H- and 2 K-encoding genes,  
343 only 43.5% of the isolates could be assigned to a serogroup. Among the O-, H- and K-  
344 encoding genes included, all were detected except O35, although frequently reported in avian  
345 colibacillosis (Nolan et al., 2020). Conversely, the high proportion of isolates with a non-

346 determined serogroup may be related to the fact that some serotypes, such as O5, O36, O81,  
347 O111, O115, O116, or O132 described in avian pathology (Nolan et al., 2020; Rodriguez-Siek  
348 et al., 2005; Schouler et al., 2012), were not included in our typing scheme. Further molecular  
349 analysis such as sequencing would help to better precise the serogroup determination of these  
350 strains. Similarly to other authors (Germon et al., 2005; Nolan et al., 2020; Rodriguez-Siek et  
351 al., 2005; Vounba et al., 2018), we detected a high proportion of O2, O18, O25, O78 and O88  
352 isolates among our colibacillosis case isolates, but O1 isolates were observed in colibacillosis  
353 from only three flocks, although O1 is frequently cited as a major serotype in APEC  
354 (Schouler et al., 2012). Possible reasons for the discrepancies between our results and other  
355 studies include the use of different methods for serogrouping. Agglutination tests depend  
356 strongly on the quality of antisera, and PCR performed with primers depend on published  
357 sequences, for which there may be sequence variants. Another source of discrepancy is the  
358 possible diversity in isolates from colibacillosis from different productions (broilers, layers or  
359 breeders), or countries. Variations in the occurrence of the different serotypes may also vary  
360 over time. In particular, the use of O1 vaccines in poultry production may have induced a shift  
361 in the most commonly found serogroups over time.

362 We used the genetic profiles of the isolates to investigate the possible origins of colibacillosis  
363 cases. Various situations were found. In some instances, all colibacillosis-associated isolates  
364 exhibited the same profile (e.g. Pf3 for the six isolates of early colibacillosis in the P5 flock,  
365 and Pf9 for the 12 late colibacillosis isolates in the P33 flock (although with two different  
366 resistance profiles)). We also observed colibacillosis cases with multiple profiles, sometimes  
367 several profiles for the same individual (e.g. five different profiles for the eight isolates  
368 obtained from four diseased chicks in the early colibacillosis cases of flock P8, or three  
369 profiles from four chickens with late colibacillosis in flock L3, with two chickens harbouring  
370 more than one profile). This diversity of situations may reveal different types of colibacillosis,



371 either due to a single, very virulent strain, multiple mildly virulent strains acting  
372 synergistically or consecutive to poor production conditions, leading to infections caused by  
373 various opportunistic strains. Therefore, due to the possible presence of several strains in a  
374 colibacillosis case, it is necessary to characterize several isolates when an antimicrobial  
375 treatment or preparation of an autovaccine are needed, as observed in humans (Levert et al.,  
376 2010).

377 In some cases, identical profiles were detected in colibacillosis cases and DOCs, and,  
378 remarkably, sometimes in different flocks, e.g. Pf3 in DOCs and early colibacillosis cases in  
379 two flocks (P5 and P8, belonging to the same production company, and sharing the same feed  
380 supplier, hatchery and poultry breed). The same profile was also sometimes observed for  
381 isolates from transport swabs, DOCs and early colibacillosis cases, e.g. Pf31 in flock L38.

382 Considering the identical profiles in DOCs from different flocks or between transport swabs  
383 and colibacillosis samples, it is tempting to speculate that the *E. coli* strains may be acquired  
384 from breeder flocks, in the hatchery or during chick transport. The transmission from breeders  
385 to their progeny has already been observed (Giovanardi et al., 2005), and that study suggested  
386 that an embryonic APEC infection possibly occurs before hatching and results in field cases  
387 of omphalitis or colibacillosis. However, we note in addition isolates sharing a profile with a  
388 colibacillosis isolate in DOCs from flocks that remained healthy (e.g. Pf7 found in  
389 colibacillosis in flocks L9 and L24, and in DOCs in flocks P11 and P25, which were not  
390 affected with colibacillosis). Similarly, profiles from transport swabs were detected in  
391 colibacillosis cases in other flocks (e.g. Pf39 in transport swabs from flock L27 which  
392 remained healthy, in flock L28, which presented colibacillosis cases with different profiles,  
393 and in early colibacillosis cases in flock L5). This finding indicates that the presence of *E. coli*  
394 in DOCs or transport swabs does not necessarily mean that the flock will be affected with

395 colibacillosis, and various factors such as the number of infected chicks or inoculum doses,  
396 and farm conditions, likely play a role in the development of colibacillosis.

397 None of the profiles observed in the farm environment isolates were detected in colibacillosis  
398 samples from the same farm, and only one farm profile was detected in a colibacillosis case  
399 (Pf125 present in the flock L24 broiler house, and in a joint sampled during a late  
400 colibacillosis episode in flock P26). However, the role of the housing environment has been  
401 demonstrated in one study (Daehre et al., 2018), with the presence of the same cephalosporin-  
402 resistant *E. coli* strain in broilers from two consecutive flocks and in the broiler house  
403 environment (boot swab and litter) before the arrival of the chicks of the second flock. The  
404 authors underlined the difficulty of eliminating (resistant) *E. coli* in the farm environment  
405 even after cleaning and disinfection. Several explanations can be offered for the absence of  
406 identical profiles in broiler houses and colibacillosis cases, with the characteristics of  
407 pathogenic *E. coli* strains differing from those of strains able to persist in the environment  
408 after cleaning and disinfection. However, it is also likely that, despite our efforts to test many  
409 isolates from different samples (walls, materials, air inlets, litter and water), we did not  
410 capture the whole diversity of the environmental (and DOC) isolates. Thus, some profiles,  
411 such as Pf9 (12 late colibacillosis isolates in flock P33), were observed only in colibacillosis  
412 lesions in a single flock, hampering the possibility of finding the origin of the contamination,  
413 although 19 other isolates from DOCs, transport swabs and the farm environment had been  
414 characterized for this flock. Similar situations include, for instance, the L28 flock (six early  
415 colibacillosis isolates with Pf18, but all six isolates from DOCs, transport swabs and the farm  
416 environment differing from Pf28), or the P44 flock, with six early colibacillosis isolates of the  
417 Pf22 profile, but non-colibacillosis isolates having other profiles. It is also possible that the  
418 colibacillosis strain(s) originate from other sources (e.g. contamination via live vectors or  
419 fomites, entering the broiler house after our initial visit). In the above-mentioned broiler house

420 study (Daehre et al., 2018), despite of intensive sampling of the environment, the origin of the  
421 ESBL-AmpC contamination in some flocks could not be determined. Thus, even intensive  
422 sampling may overlook the origin of contamination, because many different sources of  
423 horizontal transmission can be encountered on a farm. Data of our study corroborates such a  
424 conclusion and reinforce the idea of testing many isolates as possible when an  
425 epidemiological investigation of colibacillosis is conducted.

426

## 427 **Conclusion**

428 In conclusion, this study offered an excellent opportunity to evaluate the genetic diversity of  
429 *E. coli* strains obtained from a large number of flocks, monitored from before chick delivery  
430 to colibacillosis outbreaks. We clearly evidenced the diverse disease conditions, with either an  
431 apparently unique genetic strain or different strains in colibacillosis lesions. The colibacillosis  
432 isolates were sometimes identical to isolates obtained from DOCs or DOC transport boxes of  
433 the same or different flocks, but the presence of isolates sharing the same profile in DOCs,  
434 transport boxes or the broiler house did not necessarily lead to a colibacillosis outbreak. Work  
435 is now in progress to get a better evaluation of the virulence of the strains based on the  
436 presence of the different genetic markers that were screened, as well as the epidemiological  
437 factors associated with a higher risk of early or late colibacillosis.

438

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440

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445

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**Table 1: numbers of samples and isolates**

**Table 2: Significant differences in the serogroups and phylogroups of isolates obtained from DOCs and early and late colibacillosis cases**

**Table 3: Origins of the 10 most frequent profiles. For each profile, the flock in which it was observed is indicated and the number of isolates is given in parentheses.**

**Figure 1: Percentage of isolates belonging to one of the 23 screened serogroups (colour) according to isolate origin**

Cose: colibacillosis; DOCs: day-old chicks; ND: serogroup not determined

**Figure 2: MCA for serogroups and phylogroups of isolates**

Early Cose: early colibacillosis, Late Cose: late colibacillosis, DOCs: day-old chicks; ND: serogroup not determined, U: phylogroup not determined

**Figure 3: Percentage of isolates belonging to the phylogroups according to their origin**

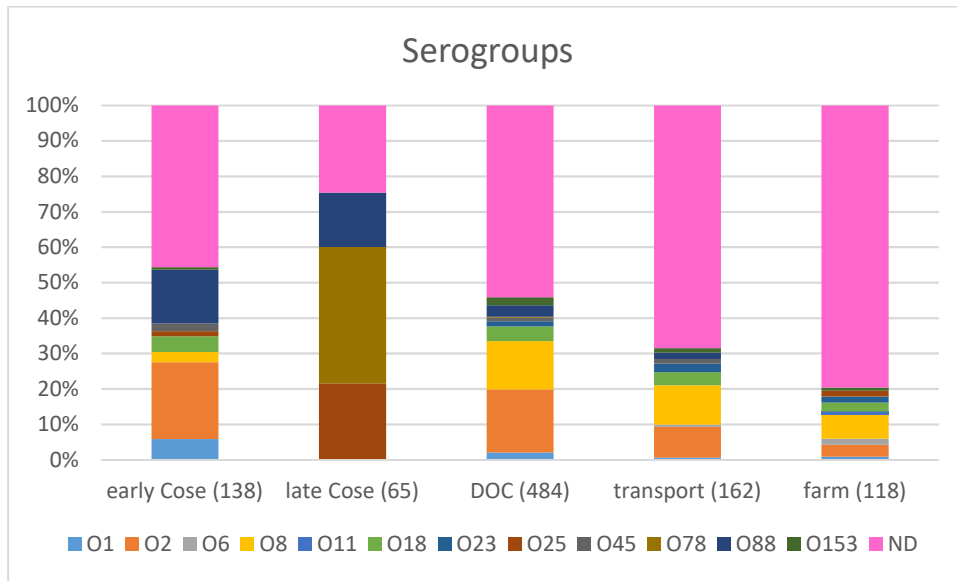
Cose: colibacillosis; DOCs: day-old chicks; ND: not determined

**Figure 4: Hierarchical clustering of the isolates according to 58 VAGs**

CP: early: colibacillosis; CT: late colibacillosis, house: farm samples; Pap: transport

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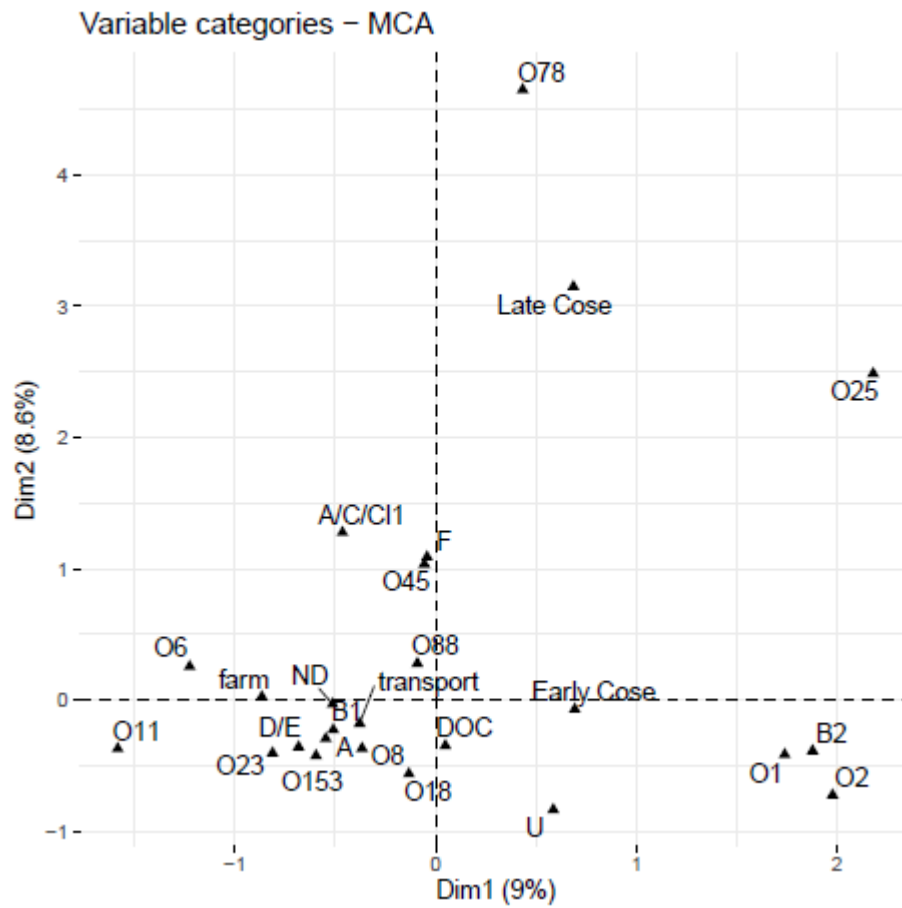
3

4 **Figure 1: Percentage of isolates belonging to one of the 23 screened serogroups (colour)**

5 **according to isolate origin**

6 Cose: colibacillosis; DOCs: day-old chicks; ND: serogroup not determined





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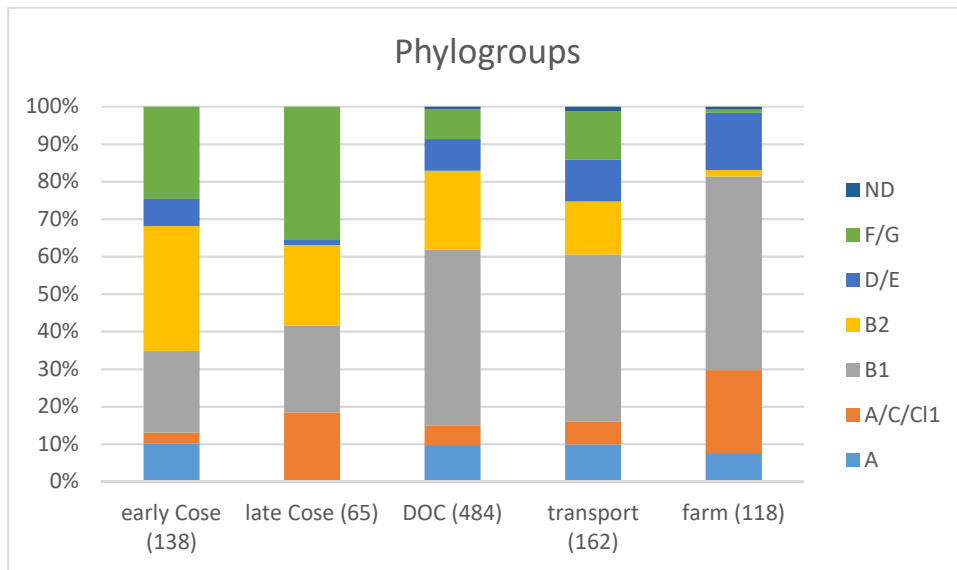
2 **Figure 2: MCA for serogroups and phylogroups of isolates**

3 Early Cose: early colibacillosis, Late Cose: late colibacillosis, DOCs: day-old chicks; ND:

4 serogroup not determined, U: phylogroup not determined

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3 **Figure 3: Percentage of isolates belonging to the phylogroups according to their origin**

4 Cose: colibacillosis; DOCs: day-old chicks; ND: not determined



3 **Figure 4: Hierarchical clustering of the isolates according to 58 VAGs**

4 CP: early: colibacillosis; CT: late colibacillosis, house: farm samples; Pap: transport

**Table 1: numbers of samples and isolates**

Context	Samples sent to laboratory	Number of isolates per flock	Total number of isolates analysed
At placement	10 DOCs 6 house and paper swabs	Up to 20 isolates from yolk or liver Up to 12 isolates	484 280
Early colibacillosis	10 chicks	4-16 isolates from yolk, liver, spleen, heart, joints or air sacs from at least 4 chicks	138
Late colibacillosis	6 chickens	4-20 isolates from liver, spleen, heart, joints or air sacs from at least four chickens	65

**Table 2: Significant differences in the serogroups and phylogroups of isolates obtained from DOCs and early and late colibacillosis cases**

	Not significantly different	Significantly different*
Early colibacillosis <i>versus</i> late colibacillosis	O8, O88 B1, B2, D/E, F/G	SND <sup>E</sup> , O2 <sup>E</sup> , O25 <sup>L</sup> , O78 <sup>L</sup> A <sup>E</sup> , A/C/CII <sup>L</sup>
Early colibacillosis <i>versus</i> day-old chicks	SND, O2, O78 A, A/C/CII, D/E	O8 <sup>D</sup> , O25 <sup>E</sup> , O88 <sup>E</sup> B1 <sup>D</sup> , B2 <sup>E</sup> , F/G <sup>E</sup>
Late colibacillosis <i>versus</i> day-old chicks	B2	SND <sup>D</sup> , O2 <sup>D</sup> , O8 <sup>D</sup> , O25 <sup>L</sup> , O78 <sup>L</sup> , O88 <sup>L</sup> A <sup>D</sup> , A/C/CII <sup>L</sup> , B1 <sup>D</sup> , D/E <sup>D</sup> , F/G <sup>L</sup>

SND: serogroup not determined

\*For each serogroup and phylogroup, the superscript letter indicates the origin (E: early colibacillosis, L: late colibacillosis, D: DOCs) with the highest percentage

**Table 3: Origins of the 10 most frequent profiles. For each profile, the flock in which it was observed is indicated and the number of isolates is given in parentheses.**

Profile	# ISOLATES	# FARMS	cases	Early colibacillosis	Late colibacillosis	DOCs	Transport swabs	Farm swabs
Pf1	44	9	L31 (1)			L12 (5)	L12 (1)	
						L34 (4)	L39 (1)	
						P9 (16)	P9 (2)	
						P11 (1)		
						P14 (6)		
						P39 (3)		
						P44 (4)		
Pf2	19	1				L23 (17)	L23 (2)	
Pf3	17	2	P5 (6)	P8 (3)		P5 (7)		
						P8 (1)		
Pf4	15	6				L27 (2)		P38 (2)
						L33 (5)	L33 (2)	
						L36 (1)		
						P18 (1)		

				P38 (2)	P40 (1)
	14	7	L9 (1)	P19 (1)	L18 (2)
Pf5			L17 (3)		P2 (4)
			L24 (2)		P15 (1)
Pf6	12	1			L4 (12)
	12	4	L9 (1)	L9 (2)	L14 (1)
Pf7			L14 (2)	P11 (1)	P11 (1)
				P25 (4)	
	12	4	L31 (6)	P15 (1)	
Pf8				P28 (1)	P28 (1)
				P30 (3)	
Pf9	12	1		P33 12	
Pf10	11	1			P42 (11)