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

Sabine Delannoy[a], Catherine Schouler[b], Rozenn Souillard[c], Léna Yousfi[a], Laetitia Le Devendec[c], Camille Lucas[c], Stéphanie Bougeard[c], Alassane Keita[c], Patrick Fach[a], Pascal Gallot[d], Loïc Balaine[c], Julie Puterflam[d*], and Isabelle Kempf[a*]

*contributed equally to this work

[a]Université Paris-Est, ANSES, Food Safety Laboratory, Platform IdentyPath, 94701 Maisons-Alfort, France

[b] ISP, INRAE, Université de Tours, UMR 1282, 37380, Nouzilly, France

[c]ANSES, Ploufragan-Plouzané-Niort Laboratory, France

[d]ITAVI, Ploufragan

Corresponding author: Isabelle Kempf

isabelle.kempf@anses.fr

ANSES Laboratoire de Ploufragan-Plouzané-Niort

Unité Mycoplasmologie, Bactériologie et Antibiorésistance

Zoopole Les Croix

22440 Ploufragan

France

Abstract

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

Keywords

Broilers - Colibacillosis - Strain - Diversity

Introduction

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

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

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

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

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

Materials and methods

Farms and flocks

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

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

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

E. coli isolation

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

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

High-throughput PCR

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

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

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

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

Antimicrobial susceptibility testing

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

Statistical methods

Statistical methods

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

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

Results

Number of colibacillosis and number of strains

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

Diversity of strains

All 967 isolates carried *cdgR*, confirming the *E. coli* species identification. The *ompA* and the *papG* allele I genes were found in all isolates, but neither the *focG*, LT, STa, STb, *stx*1 nor the *stx*2 genes were found in any of the isolates. These genes were thus excluded from the genetic profiling study.

Serogroups

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

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

The distribution of the serogroups varied with the source of isolation (Table 2). The numbers of O2 and non-O2 serogroups varied between isolate origins, except between DOCs (18% of O2 isolates) and early colibacillosis cases (22% of O2 isolates), between farm samples (3% of O2 isolates) and late colibacillosis cases (0% of O2 isolates) and between farm (3% of O2 isolates) and transport (9% of O2 isolates) samples. The percentage of O8 isolates was significantly higher in DOCs (14%) than in colibacillosis cases (3% for early ($p= 0.0002$) and 0% for late colibacillosis ($p= 0.0003$)) and more O8 isolates were detected in transport samples (11%) than in early colibacillosis cases (3%) ($p= 0.007$). Regarding the O25 serogroup, MCA showed that late colibacillosis isolates belonged significantly more often to the O25 serogroup (22% of O25 isolates, originating from three flocks) than the isolates from other origins (maximum of 2% of O25 isolates, $p<10^{-5}$) (Figure 2). In all, 38.5% of late colibacillosis isolates belonged to the O78 serogroup, compared with less than 0.2% in the other isolate groups ($p<2x10^{-13}$) (Figure 2). The distributions of O88 and non-O88 were not significantly different between early and late colibacillosis isolates (15% of O88 isolates for each), and there were significantly more O88 isolates in colibacillosis cases than in farm isolates ($p<2.10^{-5}$), transport isolates ($p<0.0003$) and DOCs ($p<2x10^{-5}$) (less than 3% for each).

Phylogroups

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

Significantly fewer late colibacillosis isolates belonged to phylogroup A (0% compared with more than 8% for isolates from other origins, $p<0.03$) (Table 2). Isolates from early or late colibacillosis cases belonged significantly less frequently to phylogroup B1 compared with isolates from other origins (22% and 23%, respectively, compared to a minimum of 44% for other groups of isolates, $p<4x10^{-5}$ for early colibacillosis, $p<0.003$ for late colibacillosis). There were significantly more isolates belonging to the B2 phylogroup among those obtained from early colibacillosis (33%) than those from DOCs (21%) ($p=0.003$), transport samples (14%) ($p=9x10^{-5}$) or farm samples (2%) ($p=3x10^{-12}$), and significantly fewer isolates from farm samples belonged to this phylogroup compared with all other origins ($p<0.0002$). Late colibacillosis isolates belonged less frequently to phylogroup D/E (1.5%) (Figure 2) than isolates from DOCs, transport and farm swabs (respectively 8.5%, 11% and 15%, ($p<0.05$)). Significantly more isolates from colibacillosis cases fell in phylogroup F/G (25% and 35% for early and late colibacillosis respectively, $p<0.01$ and $p<0.0002$ respectively) than isolates of other origins; furthermore, only 1% of farm isolates belonged to this phylogroup, compared with percentages of higher than 8% for the other origins ($p<0.003$).

Table 2 summarizes the significant and non-significant differences of the serogroup and phylogroup distributions between DOC and early and late colibacillosis isolates. MCA also revealed associations between serogroup O1 and phylogroup B2 ($p=5x10^{-9}$), and serogroup O2 and phylogroup B2 ($p= 8x10^{-7}$).

Diversity of genetic profiles

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

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

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

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

The 21 cases of early colibacillosis, for which 138 isolates were available, yielded 65 profiles, with 34 singletons. One to 12 (mean 6.6) isolates per case were studied. For each case, one to eight profiles were detected, with a mean of 3.5 profiles per case. From each chick, we 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.

Hierarchical clustering of the isolates according to 58 VAGs (excluding serogroup and phylogroup information) as part of the heatmap analysis identified two main clusters (Figure 4). Interestingly, these clusters could be clearly distinguished by their phylogroup. One cluster (on the right-hand side of the heatmap) contained all of the isolates from phylogroups B2, F/G, E/Cl1/U and some isolates from phylogroup D/E, whereas the other cluster grouped all of the isolates from phylogroups A, B1, A/C/Cl1, and Cl1/Cl2/U and the remaining isolates from phylogroup D/E. These two clusters appeared to be mainly differentiated by the presence/absence pattern of the cluster of VAGs at the bottom of the heatmap, i.e. *tsh*, *vat*, *fyuA*, *YqiC*, frz_{orf4}, *tkt1*, *aec4*, *csgA2* and *ibeA*.

One sub-cluster of the A/B1 cluster (located on the right-hand side of the cluster on the heatmap) clearly differed by the absence of the VAGs located at the top of the heatmap, i.e. the APEC ColV plasmid-associated VAGs. Interestingly, none of the isolates from this sub-cluster lacking the APEC ColV plasmid were isolated from a colibacillosis case.

Other than this sub-cluster, no other clear associations between VAG and isolate origin were detected.

Antimicrobial susceptibility profiles

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

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

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

Regarding serogroups, although we included primers for 12 O-, 5 H- and 2 K-encoding genes, only 43.5% of the isolates could be assigned to a serogroup. Among the O-, H- and K-encoding genes included, all were detected except O35, although frequently reported in avian 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 occurence 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

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

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

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

Conclusion

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

Declarations of interest: none

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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

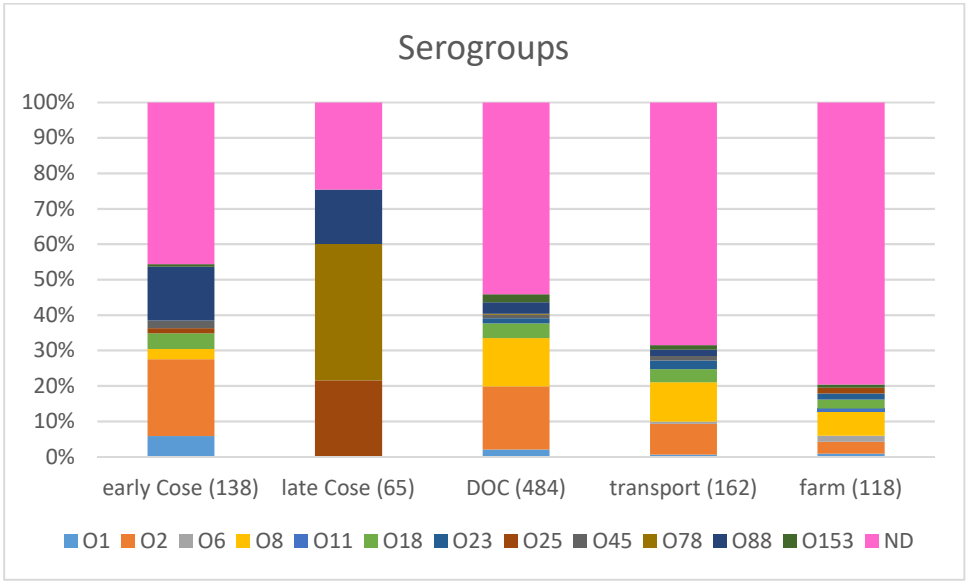
Early Cose: early colibacillosis, Late Cose: late colibacillosis, DOCs: day-old chicks; ND: serogroup not determined, U: phylogroup not determined

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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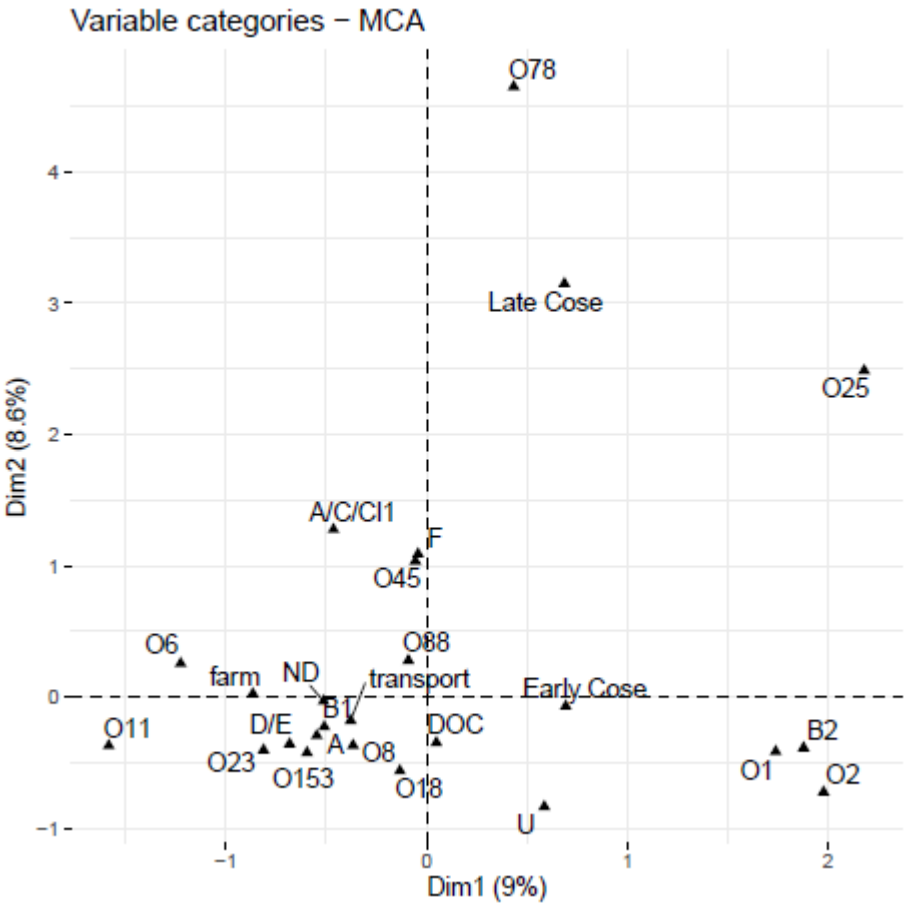

Figure 2: MCA for serogroups and phylogroups of isolates

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

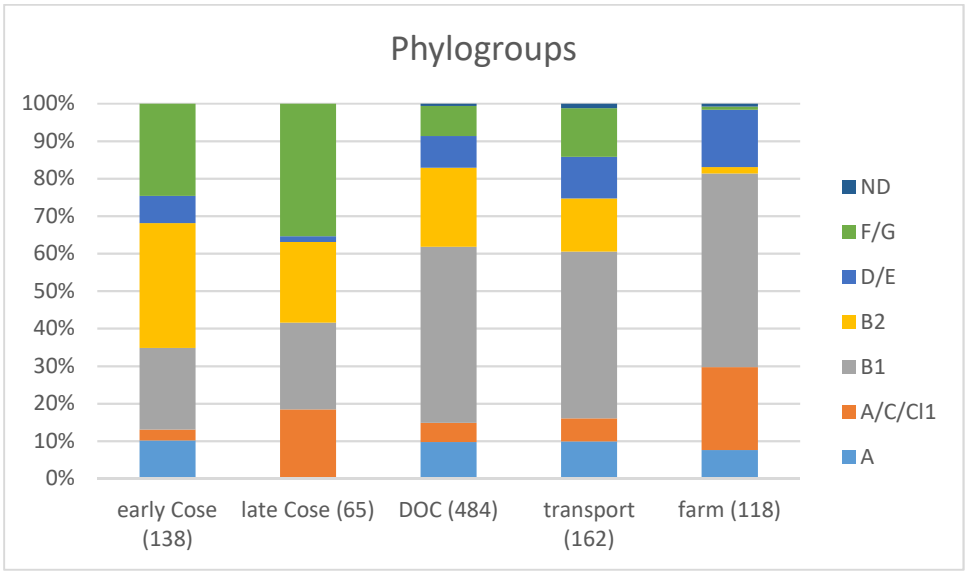

Figure 3: Percentage of isolates belonging to the phylogroups according to their origin

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

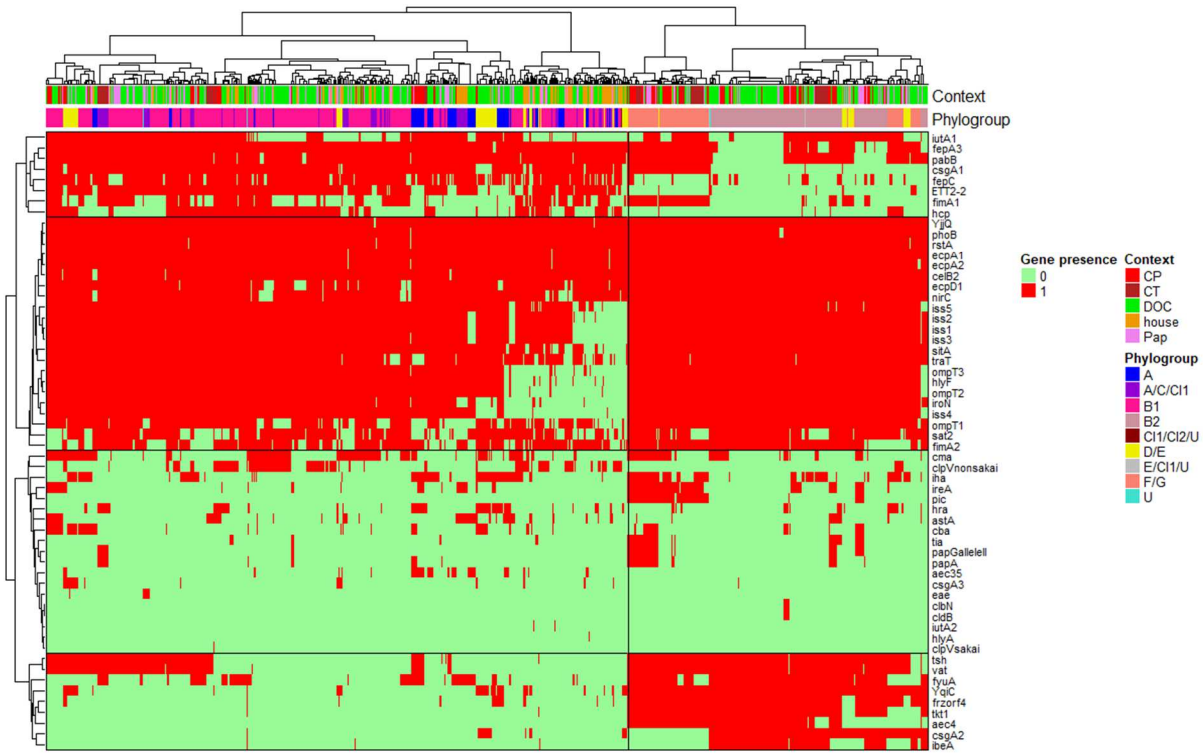

Figure 4: Hierarchical clustering of the isolates according to 58 VAGs

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

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 *versus* late colibacillosis	O8, O88	SND^{E}, $O2^{E}$, $O25^{L}$, $O78^{L}$
	B1, B2, D/E, F/G	A^{E}, $A/C/ClI^{L}$
Early colibacillosis *versus* day-old chicks	SND, O2, O78	$O8^{D}$, $O25^{E}$, $O88^{E}$
	A, A/C/ClI, D/E	$B1^{D}$, $B2^{E}$, F/G^{E}
Late colibacillosis *versus* day-old chicks		SND^{D}, $O2^{D}$, $O8^{D}$, $O25^{L}$, $O78^{L,}$ $O88^{L}$
	B2	A^{D}, $A/C/ClI^{L}$, $B1^{D}$, D/E^{D}, F/G^{L}

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

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