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Efficacy of passive immunization in broiler chicks via an inactivated *Escherichia coli* autogenous vaccine administered to broiler breeder hens

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13 ## ABSTRACT

14 Avian pathogenic *Escherichia coli* (APEC) cause extra-intestinal infections called colibacillosis, which is
15 the dominant bacterial disease in broilers. To date, given the diversity of APEC strains and the need for
16 an acceptable level of protection in day-old chicks (DOCs), no satisfactory commercial vaccine is
17 available. As part of a French nationwide project, we selected three representative strains among
18 several hundred APEC that cause colibacillosis disease. We first performed experiments to develop
19 colibacillosis *in vivo* models, using an inoculum of $3x10^7$ CFU of each *E. coli* strain per chick. Two APEC
20 strains (19-381 and 19-383-M1) were found to be highly virulent for DOCs, whereas the third strain
21 (19-385-M1) induced no mortality nor morbidity.

22 We then produced an autogenous vaccine using the 19-381 and 19-383-M1 APEC strains and a passive
23 immunization trial was undertaken. Specific-pathogen-free Leghorn hens were vaccinated twice two
24 weeks apart, the control group receiving a saline solution. The vaccinated and control hens exhibited
25 no clinical signs and egg production and fertility of both groups were similar. Fertile eggs were collected
26 for two weeks after the second vaccination and DOCs were obtained. After challenge with each APEC
27 (19-381 and 19-383-M1), DOCs appeared to be partially protected from infection with the 19-383-M1
28 strain, with 40% mortality instead of 80% for the non-vaccinated chicks. No protection was found when
29 the chicks were challenged with the 19-381 strain. Now, further work is needed to consider some
30 aspects: severity of the pathogen model, persistence of the protection, number of APEC strains in the
31 autogenous vaccine, choice of adjuvants and heterologous protection of the vaccine made from strain
32 19-383-M1.

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RESEARCH HIGHLIGHTS

- Three APEC strains were characterized and selected to develop in vivo models of colibacillosis
- A bivalent autogenous vaccine was produced and a passive immunization trial was carried out
- Protection of chicks was demonstrated when challenged with the 19-383-M1 APEC strain (homologous challenge)
- Further work is needed in particular to evaluate the protection against heterologous challenge

KEYWORDS

Escherichia coli, broiler, day-old-chicks, pathogen model, challenge, mortality, autogenous vaccine, passive immunization

50 ## Introduction
51
52 Avian pathogenic *Escherichia coli* (APEC) cause extra-intestinal infections called colibacillosis, which –
53 even more today – is the dominant bacterial disease on *Gallus gallus* farms (broilers and laying hens,
54 in particular). Colibacillosis causes high mortality, high morbidity and high rates of carcass
55 condemnations at the slaughterhouse; the resulting decrease in production leads to significant
56 economic losses for the farmer. It also causes salpingitis and septicaemia in breeders. Moreover, APEC
57 may be a potential foodborne zoonotic pathogen and a source or reservoir of extra-intestinal infections
58 in humans (Moulin-Schouleur *et al.*, 2007; Mellata, 2013; Liu *et al.*, 2018). To control the disease,
59 antibiotics such as beta-lactams, colistin and fluoroquinolones are used, most often by oral route and
60 administered in the drinking water. These treatments constitute a proven risk of selection and
61 dissemination of antimicrobial resistance genes and resistant bacterial strains, also leading to public
62 health concern.

63 APEC harbour numerous virulence factors that cause colibacillosis. Two recent publications
64 (Christensen *et al.*, 2021; Kathayat *et al.*, 2021) inventoried these virulence factors, which include
65 adhesins, invasins, protectins, iron acquisition systems, toxins, a quorum-sensing system,
66 transcriptional regulators, genes associated with metabolism; this list is not exhaustive. Moreover,
67 APEC produce extracellular polymeric substances with more than 500 different proteins that may
68 interact with the host, combined with other bacteria, *E. coli* or others (Eboigbodin & Biggs, 2008).
69 Another, more recent paper (Delannoy *et al.*, 2021) evaluated the genetic diversity of *E. coli* strains
70 isolated from 80 broiler flocks, monitored from before chick placement to colibacillosis outbreaks. The
71 *E. coli* isolates were characterized using high-throughput qPCR to screen genetic markers related to 23
72 serogroups, five phylogroups and 66 virulence factors and determine genetic profiles. In addition to
73 other findings, the study highlighted the huge diversity among avian *E. coli* with, for example, some
74 flocks for which day-old chicks (DOCs) harboured the genetic profile of colibacillosis cases identified in
75 other flocks, but nevertheless remained healthy.

76 Beyond the concern related to colibacillosis, the strong regulatory, scientific and societal
77 pressure to reduce the use of antibiotics in poultry farming has led to search for other strategies to
78 control this disease, including the development of an efficient vaccine. Despite plenty of vaccine
79 candidates demonstrating efficacy in chickens in experimental studies, only one vaccine is currently
80 commercially available in most regions worldwide (Galal *et al.*, 2018; Chrétien *et al.*, 2021). It is an
81 attenuated O78 *E. coli* strain and provides effective protection against a challenge with the O78 wild
82 strain (Koutsianos *et al.*, 2020). However, according to the summary of the characteristics of this
83 vaccine, the onset of immunity in chickens is established 2 weeks after vaccination with a reduction in
84 colibacillosis lesions. Except for a few publications and under specific conditions (Mombarg et al., 2014,
85 where the overall mortality was very high), the efficacy of the vaccine was not established to reduce
86 mortality (as shown in the summary of the product characteristics at
87 http://ircp.anmv.anses.fr/results.aspx), although mortality appears to be one of the most common
88 clinical manifestations of colibacillosis (Kemmett *et al.*, 2014). In addition, according to some studies
89 (Ghunaim *et al.*, 2014; Guabiraba & Schouler, 2015), this type of vaccine is less efficient against *E. coli*
90 heterologous strains (e.g. belonging to other serogroups or phylogroups). Therefore, this vaccine
91 sometimes suffers from limited efficacy, given the diversity of APEC strains and the difficulty of
92 obtaining a satisfactory level of protection as early as hatch time.

93 Autogenous vaccines may be one way to address and respond to this diversity of strains. They
94 are produced from APEC strains isolated from the affected flock in which the autogenous vaccine is to
95 be administered. This type of vaccine can be used either directly on target animals (Landman & van
96 Eck, 2017; Kromann *et al.*, 2021) or via a passive immunization strategy (i.e. administration of the
97 vaccine to the broiler breeders to protect the DOCs by means of antibodies transmitted by their
98 mother). Although it has yet to be demonstrated that autogenous vaccines can protect against
99 heterologous APEC strains, it is easier to adapt their composition compared to that of a commercial
100 vaccine, due to the heaviness of the drug marketing authorization process. Because of this, autogenous
101 vaccines can more easily solve the diversity of APEC strains. To our knowledge, no satisfactory vaccine

102 is available against colibacillosis in broilers and, to date, only two publications have reported possible

103 protection of DOCs using passive immunization (Rosenberger *et al.*, 1985; Heller *et al.*, 1990).

104 Here, we describe two experiments. In the first one, three APEC strains that have been

105 characterized (Delannoy*, et al.*, 2021) in terms of virulence factor content, serogroup and phylogroup

106 were used to develop *in vivo* models of colibacillosis for DOCs on two chicken breeds. The first one is

107 the ANSES' own Leghorn breed and the second one is the Ross 308 breed (Aviagen company ®), and is

108 the most common broiler breed in Europe. The use of two chicken breeds made it possible to check

109 the sensitivity of our specific-pathogen-free (SPF) Leghorn breed to the *E. coli* challenge compared with

110 that of Ross 308. This comparison allowed us to validate the use of our Leghorn SPF hens for the second

111 experiment rather than the Ross 308 SPF hens, which were not available. In the second experiment, a

112 passive immunization challenge study was then performed on broilers using a bivalent autogenous

113 vaccine administered to breeder hens.

114 Materials and methods

115 *Selection and characterization of APEC strains and preparation of mutants and inocula*

116 **Selection of three representative strains.** The experiments described in this manuscript are part of a

117 French nationwide project that has been partly recently published (Delannoy *et al.*, 2021) and for

118 which the original data involved 1050 environmental or clinical *E. coli* isolates on which 68 variables

119 were measured (i.e. phylogroup markers, serogroups and 66 virulence markers). Our first aim was to

120 select three representative strains that cause colibacillosis disease for the experimental trials;

121 therefore, statistical analyses were applied to a set of 269 *E. coli* strains from colibacillosis-diseased

122 chickens only. First, non-informative variables (i.e. same value for all the strains) were discarded.

123 Second, categorical variables were quantified and summarized using a multiple correspondence

124 analysis (MCA) (Greenacre, 1984). The most informative MCA components were used as variables

125 instead of the original ones. Third, a hierarchical clustering (Sokal & Sneath, 1963) approach was

126 applied to the selected MCA components to choose the optimal number of clusters. Fourth, clustering

127 was optimized using the k-means method (Lloyd, 1982; MacQueen, 1967) applied to the optimal
128 number of clusters. Finally, the most representative strains (i.e. closest to the mode of the cluster) of
129 each k-means cluster were identified. The R functions 'MCA' and 'hclust' of the 'FactoMiner' package
130 (Lê *et al.*, 2008), and the 'kmeans' function were used. At the end of the process, three clusters were
131 obtained and three APEC strains representative of each cluster were selected: *E. coli* 19-381 (cluster
132 1), 19-383 (cluster 2) and 19-385 (cluster 3) as shown in the figure 1**.**

133 **Preparation of mutants and inocula.** To facilitate the recovery of the strains from internal organs
134 during *in vivo* assays, spontaneous rifampicin-resistant mutants of *E. coli* 19-381, 19-383 and 19-385
135 were obtained by culturing the strains on Mueller-Hinton (MH) medium containing rifampicin (250
136 mg/L). No mutant was obtained for strain 19-381. The mutants were compared with parental strains,
137 in terms of their phylogenetic group (Clermont *et al.*, 2000) and their antimicrobial susceptibility
138 determined by a broth micro-dilution method on EUVSEC plates (Sensititre, ThermoFisher Scientific,
139 Dardilly, France). Then the *E. coli* strain (19-381) and the obtained mutants (19-383-M1 and 19-385-
140 M1) were cultured overnight in MH broth at 37°C under agitation. The cultures were centrifuged and
141 re-suspended in peptone buffer to obtain a titre of approximately $3x10^8$ colony forming units (CFU)/mL
142 for both *in vivo* trials. The objective was to be in conformity with Schouler et al. (2012) where the dose
143 inoculated per chick was about $5x10^7$ CFU. The titres were determined by plating decimal dilutions on
144 MH agar plates.

145 **Whole genome sequencing and characterization of the three strains.** Whole genome sequencing
146 (WGS) was performed on a Novaseq 6000 system with the Nextera kit. The raw reads were processed
147 using the shovill method (https://github.com/tseemann/shovill, not published yet) with the "--trim"
148 option. This method cleaned raw reads using trimmomatic (Bolger *et al.*, 2014) and assembled the
149 reads using Spades to generate contigs (Prjibelski *et al.*, 2020). The de novo contigs were then screened
150 against Megablast (Chen *et al.*, 2015) on a local nucleotide database. All contigs belonged to *E. coli*
151 strains. The contigs shorter than 200 nucleotides or with a k-mer coverage lower than 2 were filtered
152 out. The sequences were analysed using the tools from the Center for Genomic Epidemiology (CGE,

153 https://www.genomicepidemiology.org) to determine the main characteristics of the strains
154 (serotype, sequence type (ST), resistance genes and *E. coli* virulence genes) and to the ClermonTyper
155 (http://clermontyping.iame-research.center/) to determine phylogroups. The susceptibility of the
156 three strains was studied by disk diffusion assay according to the AFNOR NF U47-107 (2012).

157 ***Pathogenicity of three* E. coli *strains (19-381, 19-383-M1 and 19-385-M1) on day-old chicks***

158 The virulence of the selected APEC strains was evaluated in a lethality assay by subcutaneous
159 inoculation into DOCs and for two chicken breeds (Leghorn and Ross 308). For this experiment, we
160 used chicks hatched from 45-week-old and 40-week-old for SPF Leghorn and Ross 308 hens
161 respectively. The experimental design is shown in the table 1. Four groups were defined for each
162 chicken breed. Leghorn groups were: L-NI (non-infected), L-381 (infected with 19-381), L-383M
163 (infected with 19-383-M1), L-385M (infected with 19-385-M1). Ross 308 groups were: R-NI (non-
164 infected), R-381 (infected with 19-381), R-383M (infected with 19-383-M1), R-385M (infected with 19-
165 385-M1). The experiment was performed in accordance with French animal welfare regulations and
166 the protocol was approved by the ANSES/ENVA/UPEC Ethics Committee and the French Ministry for
167 Higher Education, Research and Innovation (APAFIS #21978-2019091215094222V1). The experiment
168 was conducted at the ANSES Ploufragan animal facilities. Four rooms and two pens per room were
169 used. The chicks were tagged with unique numbers and housed in negative pressure, air-filtered, level-
170 2 containment rooms in floor pens with wood shavings as bedding material. The DOCs were distributed
171 in the rooms in such a way to obtain similar average weights across the different experimental groups.
172 They were then inoculated subcutaneously (0.1 mL per chick, at the neck level between the two wings)
173 according to the experimental design outlined in Table 1. Daily mortality and clinical status (normal,
174 slightly depressed, prostrate) were monitored until the end of the experiment, eight days after
175 inoculation. Body weight was assessed before inoculation and at the end of the experiment. In case of
176 mortality, or for 10 live birds at the end of the experiment, colibacillosis lesions were determined and
177 liver and spleen samples were collected. All liver and spleen samples were grown on MacConkey (MC)
178 media. The samples from birds inoculated with *E. coli* 19-383-M1 or *E. coli* 19-385-M1 were grown on

179 MC media supplemented with rifampicin (250 mg/L), and those from birds inoculated with *E. coli* 19-
180 381 or *E. coli* 19-383-M1 were grown on MC supplemented with ciprofloxacin (0.25 mg/L). Hence, the
181 choice of the antibiotic used for selection was based on the resistance of the different strains. Samples
182 from the non-inoculated birds were grown on all three media. For each positive sample, one
183 randomly chosen colony was identified with an *E. coli*-specific PCR (Furet *et al.*, 2009), and its
184 phylogenetic group was determined (Clermont *et al.*, 2000; Peebles *et al.*, 2005)

185 ***APEC autogenous vaccine***

186 The autogenous vaccine used was an inactivated and adjuvanted vaccine produced by an authorized
187 laboratory (Labocea, Ploufragan, Laboratory 1879, authorization AV 0787/07 delivered by the French
188 National Veterinary Medicines Agency for the manufacture of veterinary autogenous vaccines, in
189 accordance with French regulations). The vaccine included the APEC 19-381 and 19-383-M1 strains,
190 because the 19-385-M1 strain induced no mortality nor clinical signs (see Results). The antigenic
191 fraction was composed of the corresponding whole bacterial cells, whose culture was carried out in
192 broth then in agar medium (PPLO agar base, Difco). The bacterial cells were harvested by adding 0.9%
193 NaCl physiological serum for injection (Fresenius Kabi), supplemented with 0.5% of a 37%
194 formaldehyde solution (Sigma Aldrich) to inactivate the bacteria. The concentration of bacterial cells
195 in the aqueous phase collected was between 10^8 and 10^9 CFU/mL. This aqueous phase was then
196 emulsified with an oily adjuvant ISA35 (SEPPIC) to produce an oil/water vaccine, the adjuvant
197 representing 25% (vol/vol) of the final mixture. The inactivation of the bacteria and the sterility of the
198 autovaccine were checked using tests on broths and subcultures on agar medium with a final reading
199 at 14 days, in accordance with the guidelines of the European Pharmacopoeia. Each breeder hen was
200 inoculated in the pectoral muscle with 0.3 mL of the vaccine, as described below.

201 ***Passive immunization experiment***

202 This second experiment was also performed in accordance with the same welfare and ethics
203 regulations (authorization number APAFIS #24443-2020030217596117V3). The experiment was
204 conducted at the ANSES Ploufragan animal facilities. Forty SPF Leghorn layers from ANSES Ploufragan,
205 housed in furnished cages, were randomly distributed in two groups and two separate rooms: 20 layer
206 hens were vaccinated twice at 20 and 22 weeks of age and the other 20 layer hens received a saline
207 solution at the same ages (control group). Each room housed four non-vaccinated SPF Leghorn roosters
208 as well, which were previously distributed at random. Fertile eggs were collected for two weeks after
209 the second vaccination: these eggs were thus collected from 22 to 24 week-old hens. The hens were
210 observed for clinical evaluation from the first injection until the end of egg collection. Fertile eggs from
211 both hen groups were incubated in the same incubator, but care was taken to avoid mixing them up.
212 DOCs were then obtained from vaccinated and non-vaccinated hens. Six groups of 20 chicks were then
213 randomly formed so as to obtain similar average weights (and standard deviation) in the different
214 groups (Table 2). All chicks were tagged with unique numbers. The chicks of a given group were housed
215 in negative-pressure level-2 isolators with a volume of 1.36 m^3 each (these isolators are made to order
216 for our institute). The same parameters as for the pathogenicity experiment were recorded: daily
217 mortality and clinical status until the end of the experiment, body weight before inoculation and at the
218 end of the experiment. In case of mortality or for all live birds at the end of the experiment,
219 colibacillosis lesions were determined and liver and spleen samples were collected.

220 The experimental design is presented in the table 2. Six groups of chicks were defined: non-
221 vaccinated and non-challenged (NVNC), vaccinated and non-challenged (VNC), two groups of non-
222 vaccinated and challenged (NVC 381 and NVC 383M, respectively challenged with 19-381 and 19-383-
223 M1) and two groups of vaccinated and challenged (VC 381 and VC 383M, respectively challenged with
224 19-381 and 19-383-M1). On their first day of life, the chicks from the NVC 381, NVC383 M, VC 381, and
225 VC 383M groups were challenged as described for the first experiment. Mortality, clinical status and
226 lesions of dead birds or birds sacrificed on day 9 were recorded as described above. Organs were
227 cultured as for the first trial, but only one isolate was characterized from each organ.

228 ***Statistics***

229 For both experiments, the qualitative variables (i.e. mortality, clinical status) were analysed using a
230 Chi-square test, or Fisher's exact test for small samples ($n \leq 5$). The quantitative data were analysed
231 using either an analysis of variance or a Wilcoxon test depending on the number of observations and
232 the parametric hypothesis checking. The level of significance was set to $p \leq 0.05$.

233 ***Sequences***

234 The sequences were deposited in GenBank and are available from the NCBI, BioProject
235 PRJNA795346 (Accessions SRR17934378, SRR17934377 and SRR17934376).

236 **Results**

237 ***Characterization of the three representative* E. coli *strains and mutants obtained***

238 The genetic characteristics of the colibacillosis isolates obtained were used to select three strains
239 representative of the three clusters on the basis of the characteristics (i.e. phylogroup, serogroup and
240 66 virulence markers) of the 269 colibacillosis strains. A hierarchical clustering procedure was
241 performed using 45 variables (variables with no variability were removed) and 268 strains (one atypical
242 strain was discarded).Then, a k-means method was applied with k = 3 classes. The three k-means
243 clusters, containing respectively 57, 82 and 129 strains, were the same as with those identified in the
244 hierarchical clustering (Figure 1).

245 Cluster 1 contained mostly isolates belonging to the B2 phylogroup, and to the O2:K1
246 serogroup, and possessing several plasmid virulence associated genes (e.g. *iut, ompT, tsh*), and also
247 the *ibeA* gene, absent in most isolates of Clusters 2 and 3. Cluster 2 contained isolates belonging
248 mostly to phylogroup F, and possessing the *pic, fimA1* and *ireA* genes, which are absent in most
249 isolates of Clusters 1 and 3. Cluster 3 included mostly isolates belonging to phylogroup B1 and
250 possessing, unlike most isolates of Clusters 1 and 2, the genes ETT2.2, *fepC*, *hcp* and *hra*. Finally, for

251 each class, the most representative strains (i.e. *E. coli* 19-381, 19-383 and 19-385) were selected. *E.*
252 *coli* 19-381 shared all the characteristics presented in Table 3 for strains of Cluster 1, except the *iha*
253 and *csg*A3 genes. *E. coli* 19-383 shared the characteristics of Cluster 2, except the *iutA* and *cma*
254 genes. Based on its genomic sequence, *E. coli* 19-383 was shown to belong to phylogroup G, a
255 recently described phylogroup intermediate between the F and B2 phylogroups. Thirty out of 39 of
256 the characteristics of Cluster 3 were present in *E. coli* 19-385.

257 The three selected strains had been obtained from cases of early colibacillosis (i.e. broiler
258 flocks of up to 10 days of age with a daily mortality rate higher than 0.3% and suspect clinical signs or
259 typical colibacillosis lesions). Susceptibility tests showed that *E. coli* 19-381 was resistant to
260 sulfamethoxazole, trimethoprim, tetracycline, ciprofloxacin and ampicillin; *E. coli* 19-383 was resistant
261 to sulfamethoxazole, trimethoprim, tetracycline, ciprofloxacin, ampicillin and chloramphenicol and *E.*
262 *coli* 19-385 was resistant to sulfamethoxazole and tetracycline.

263 We were able to obtain rifampicin-resistant mutants for *E. coli* 19-383 and *E. coli* 19-385, but not for
264 *E. coli* 19-381. The two mutants belonged to the same phylogenetic group as their parental strains and
265 susceptibility testing showed that, in addition to rifampicin resistance, the mutants *E. coli* 19-383-M1
266 and *E. coli* 19-385-M1 were resistant to the same antibiotics as their parental strains. Thus the *in vivo*
267 experiments were performed with *E. coli* 19-381 and the mutants *E. coli* 19-383-M1 and *E. coli* 19-385-
268 M1 .

269 Results of the WGS of these three strains are presented in Table 4. The *E. coli* 19-381, 19-383-
270 M1 and 19-385-M1 strains belonged respectively to serotypes O50/O2:H5:K1, O24:H4 and O86:H51,
271 and to ST140, ST117 and ST155. Phylogroups determined by PCR were confirmed by WGS. Based on
272 the various virulence-associated genes screened for using qPCR and the CGE web tool, *E. coli* 19-381
273 and 19-383-M1 had a high number of virulence-associated genes (39 each), whereas 29 virulence-
274 associated genes were detected in *E. coli* 19-385-M1. The first two strains had the ciprofloxacin
275 resistance mutation in the *gyrA* gene (S83L) and harboured genes encoding resistance to beta-lactams,

276 tetracyclines, sulfonamides, trimethoprim and aminoglycosides. *E. coli* 19-383-M1 also had resistance

277 genes to macrolides and chloramphenicol. *E. coli* 19-385-M1 had only resistance genes to beta-lactams

278 and tetracyclines.

279 ***Pathogenicity experiment***

280 Numbers of bacteria inoculated per chick were 3.2 x10^7, 3.4 x10^7 and 2.6 x10^7 CFU for *E. coli* 19-381,

281 19-383-M1 and 19-385-M1, respectively.

282 Observed mortality and clinical status are given in Table 5. The virulence profile for chicks of

283 the three APEC strains was quite different. Mortality in Ross 308 was 0% (R-NI and R-385M groups),

284 84% (R-383M group) and 100% (R-381 group), all chicks dying as early as the day following inoculation

285 for R-381. The surviving chicks (R-383M) presented a significantly more severe clinical state compared

286 with the control group until Day 4 (D4), the difference in score distribution being non-significant

287 thereafter. Regarding the Leghorn chicks, mortality was 0% (L-NI and L-385M), 52% (L-383M) and 100%

288 (L-381), all chicks dying before D4 for L-381. The surviving chicks (L-383M) presented a significantly

289 more severe clinical state compared with the control group until D4, the difference being non-

290 significant thereafter. Thus, the two chicken breeds were susceptible to colibacillosis, although the

291 mortality rate was significantly higher for Ross 308 than for Leghorns for APEC 19-383-M1.

292 For the Ross 308 chicks, the average body weight at the end of the study was 173.0 g (25 chicks;

293 standard deviation (SD), 25.5 g) for the R-NI group, 123.5 g (4 chicks; SD, 17.2 g) for the R-383M group

294 and 165.0 g (25 chicks, SD, 23.5 g) for the R-385M group. The average weight was significantly higher

295 in the R-NI and the R-385M groups than in the R-383M group ($p = 0.001$, ANOVA). The average weights

296 of the R-NI and R-385M groups were not significantly different. Regarding the Leghorn chicks, the

297 average body weight at the end of the study was 92.2 g (25 chicks; SD, 7.2 g) for the L-NI group, 79.4 g

298 (12 chicks; SD, 8.3 g) for the L-383M group and 89.2 g (24 chicks; SD, 8.5 g) for the L-385M group,

299 respectively. This average weight was significantly higher in the L-NI ($p<0.001$) and the L-385M

300 (p=0.002) groups than in the L-383M group. The average weights of the L-NI and L-385M groups were
301 not significantly different.

302 Regarding post-mortem findings, no lesions were observed in the control group. When death
303 was sudden, there was generalized congestion. Conversely, when the chicks survived a few days,
304 pericarditis and perihepatitis were observed. Within each group, the numbers of positive liver and
305 spleen samples on the different media tested (MC, MC-Rif and MC-Cip) were not significantly different
306 (p>0.05). All cultures from the control non-inoculated group were negative. Overall, for the group
307 inoculated with *E. coli* 19-381, all analysed chicks were positive. For the group inoculated with *E. coli*
308 19-383-M1, 19 out of 25 Leghorn and 24 out of 25 Ross 308 chicks were positive (p>0.05). For the
309 group inoculated with *E. coli* 19-385-M1, 4 out of 10 Leghorn and 3 out of 10 Ross 308 chicks were
310 positive (p>0.05). The numbers of positive chicks in the inoculated groups were significantly different
311 from each other, with the highest proportion in the 19-381 inoculated group (32 positive chicks out of
312 32 analyzed), the lowest proportion in the 19-385-M1 group (7 positive chicks out of 20 analyzed), and
313 the 19-383-M1 group showing an intermediate proportion (43 positive chicks out of 50 analyzed). All
314 isolates (153 from MC, 142 from MC-CIP and 85 from MC-Rif) belonged to the expected phylogroups.

315 ***Passive immunization experiment***

316 No clinical signs were observed in the vaccinated or the control hens. Moreover, egg production and
317 fertility of both groups were similar. For this experiment, the dose of *E. coli* (in CFU in 0.1 ml volume)
318 that was administered per chick was 1.2×10^7 and 3.2×10^7 for strains 19-381 and 19-383-M1,
319 respectively.

320 Results are presented in Table 6. No mortality nor clinical signs occurred in the control groups
321 (negative control and vaccine control). No significant difference was observed, either in mortality (90%
322 versus 95%) or in the clinical status over time, between the non-vaccinated and vaccinated chicks that
323 were challenged with the APEC 19-381 strain. Conversely, for chicks that were challenged with the
324 APEC 19-383-M1 strain, the mortality rate was significantly lower in the vaccinated group (40%) than

325 in the non-vaccinated group (80%), as shown in Figure 2 ($p<0.05$). However, no significant differences
326 were observed between groups for their clinical status over time.

327 For the comparisons that were carried out (vaccinated *vs* non-vaccinated groups that were
328 challenged with either *E. coli* strain 19-381 or 19-383-M1), no significant differences were noted
329 between the different groups regarding body weight at day 9. Regarding post-mortem findings, the
330 results obtained during the first experiment were confirmed: generalized congestion in case of rapid
331 death or pericarditis and/or perihepatitis for chicks that survived several days.

332 Similar to the first trial, results obtained on the three media were not significantly different
333 ($p>0.05$). Overall, most samples from challenged chicks, vaccinated or not, were found positive by
334 culture. All birds collected up to and on day 6 were positive. Thereafter, only two birds of the NVC381
335 and one from the VC383 group were negative. All tested isolates (46 from the MC media, 40 from the
336 MC-CIP media and 23 from the MC-Rif media) belonged to the expected phylogroup.

337 **Discussion**

338 The genes and mutations detected in the genomes of the three strains were consistent with the
339 susceptibility phenotypes, i.e. the mutation S83L in the *gyrA* gene of *E. coli* 19-381 and 19-383
340 leading to quinolone resistance, and the presence of the following resistance genes: $bla_{\text{TEM-1B}}$ for
341 resistance to ampicillin (19-381), *tet*(A) or *tet*(B) for resistance to tetracycline (the three strains), *sul1*
342 or *sul2* for resistance to sulfamethoxazole (the three strains), *dfrA1* for resistance to trimethoprim
343 (19-381 and 19-383) and *catA1* for resistance to chloramphenicol (19-383). *E. coli* 19-381 and 19-383
344 also had *strA, strB* or *aadA1,* which encode resistance to streptomycin, but this antibiotic was not
345 tested.

346 *E. coli* 19-381 is a O50/O2:K1 :H5, B2, ST140 isolate. Indeed *E. coli* strains belonging to this ST
347 have been reported among the most prevalent isolated from avian colibacillosis and may be involved
348 in human diseases: Mehat *et al.* (2021) reported that the O1 and O2 serotypes, which represent with

349 O78 80% of APEC isolates, belong to a lineage including ST-95, ST140 and ST428/ST429 strains. Zhu
350 Ge *et al.* (2014) studied the APEC isolate IMT5155 (O2:K1:H5; ST140), isolated from a diseased
351 chicken in Germany in 2000, and showed that it shared close relationship with ST95 APEC O1:K1 and
352 human ExPEC O18:K1 strains. *E. coli* 19-383-M1 belongs to serotype O24:H4, phylogroup G. Indeed,
353 phylogroup G is composed of one main ST complex, STc117, a poultry-associated lineage with
354 extensive resistance to antibiotics (Clermont *et al.*, 2019). *E. coli* 19-383M1 carries most of the
355 virulence genes frequently present in strains of STc117 (Clermont*, et al.*, 2019). ST117 APEC were
356 previously found to be implicated in large outbreaks of colibacillosis in both parents and broilers in
357 Nordic countries (Ronco *et al.*, 2017). Thus, the two strains selected to prepare the autogenous
358 vaccine are clearly important poultry pathogens. *E. coli* 19-381 and 19-383 were highly virulent for
359 DOCs, because they induced a high percentage of mortality within a few days. Both strains contain a
360 high number of virulence-associated genes. In particular, they have five (19-381) or four (19-383) of
361 the predictors of pathogenicity (*iss, iutA, hlyF, iroN* and *ompT*) proposed by Johnson (Johnson *et al.*,
362 2008) and, according to this scheme, they would be classified as virulent. Regarding the *E. coli* 19-385
363 strain, in our experimental conditions, it was not virulent for DOCs. Interestingly, this strain contains
364 the plasmid virulence-associated genes *hlyF, iroN, iss* and *ompT*, and would have been classified as
365 virulent according to the scheme of Johnson (Johnson *et al.*, 2008), whereas according to the
366 diagnostic strategy proposed by Schouler *et al.* (2012) *E. coli* 19-381 would be classified as virulent
367 but not *E. coli* 19-383 and *E. coli* 19-385.

368 Regardless of the chicken strain used in the first experiment, challenge with APEC 19-381
369 resulted in 100% mortality four days post-inoculation at the latest. Mortality during the second
370 experiment was at least 90%. This APEC strain probably has a lower lethal dose than the 19-383-M1
371 strain. For the *E. coli* strains that were pathogenic, mortality rate or pattern was different for the Ross
372 308 and for the Leghorn breeds. Thus, significantly higher mortality was observed in the R-383M (Ross
373 308 chicks infected with 19-383-M1 strain) group (84%) than in the L-383M (Leghorn chicks infected
374 with 19-383-M1 strain) group (52%). Regarding the APEC 19-381 strain, although the mortality rate

375 was 100% for both chicken breeds, all R-381 chicks (Ross 308, infected with 19-381 strain) died before
376 D1, whereas mortality was slightly delayed for the L-381 chicks (Leghorn, infected with 19-381 strain),
377 all of them dying before D4. This difference in susceptibility of the two chicken breeds is in line with
378 other studies (Yunis *et al.*, 2000; Ask *et al.*, 2006) and may be due to their different growth rates.
379 Likewise, several studies have demonstrated an inverse relationship between growth rate and
380 resistance to colibacillosis (Yunis*, et al.*, 2000; Yunis *et al.*, 2002); the average daily weight gain was 13
381 g/day and 5 g/day in the Ross 308 R-NI (non-infected) and Leghorn L-NI (non-infected) groups,
382 respectively.

383 The mortality induced by the APEC 19-383-M1 strain in Leghorn chicks was different between
384 the two experiments (52% mortality in experiment 1 *vs* 80% in experiment 2). This difference may be
385 related to breeder age. The layers were 45 weeks old in experiment 1 versus 22 to 24 weeks old in
386 experiment 2. Mortality during the first week of life is higher in chicks from young hens, thus indicating
387 a greater fragility of these chicks (Pedroso *et al.*, 2005; Peebles*, et al.*, 2005). In the present study, we
388 successfully demonstrated the partial protection of chicks through the vaccination of breeder hens.
389 Our results show that APEC 19-383-M1 caused 40% mortality in chicks from hens that were vaccinated,
390 compared with 80% for chicks from hens that were non-vaccinated. This protection against
391 homologous APEC strains is in line with the two passive immunization studies that have been published
392 to date for chickens (Rosenberger*, et al.*, 1985; Heller*, et al.*, 1990). According to these authors, the
393 passive immunization process is linked to the level of maternally derived antibodies following hen
394 vaccination. In addition, there is a correlation between the hen's antibody titre and percentage of
395 survival of her progeny.

396 Due to the diversity of APEC strains and the need for an acceptable level of protection as early as
397 hatch time, no satisfactory commercial vaccine is currently available. Under these circumstances,
398 recent publications have highlighted new knowledge on APEC colonization and the usefulness of
399 autogenous vaccines. Today, most scientists agree that *E. coli* colonizing DOCs may originate from their

400 mother hens (Poulsen *et al.*, 2017). These studies estimate horizontal spreading of *E. coli* in the hatcher
401 to be 95% in comparison to the 5% of genuine vertical transfer. On the other hand, a recent paper
402 (Lozica *et al.*, 2021) investigated the effect of autogenous *E. coli* vaccines on the prevalence of 84
403 virulence-associated genes in *E. coli* isolated from four and five consecutive flocks at two broiler
404 breeder farms. Results indicate that continuous application of autogenous vaccines led to lower
405 genetic diversity of *E. coli* housekeeping genes, even if no such effect was observed for the diversity of
406 virulence genes. The successful use of autogenous vaccines, including through passive immunization,
407 will require a rational and judicious choice of the included APEC strains: characterization of the strains,
408 determination of their pathogenicity using modern methods (machine learning, etc.), to establish a
409 link between clinical outbreaks and other factors, including management (Christensen*, et al.*, 2021).

410 **Conclusion**

411 In this study, for our experimental trials, we selected three representative strains (19-381, 19-383-M1
412 and 19-385-M1) among several hundred APEC strains that cause colibacillosis disease. We first
413 performed pathogenicity experiments to develop colibacillosis in *in vivo* models. Two APEC strains (19-
414 381 and 19-383-M1) were found to be highly virulent for DOCs, whereas the 19-385-M1 strain induced
415 no mortality or morbidity. We then developed a bivalent autogenous vaccine (19-381 and 19-383-M1)
416 and carried out a passive immunization trial. We demonstrated partial protection of chicks when
417 challenged with the 19-383-M1 strain. Further work is needed to assess the effect of the APEC
418 challenge dose inoculated, hen age, the persistence and mechanisms of protection by passive
419 immunization, the number of APEC strains to use in the autogenous vaccine, the judicious choice of
420 adjuvants and the heterologous protection of the vaccine made from strain 19-383-M1.

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Declaration of Competing Interest

The authors report no declarations of interest.

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566 **Table 1.** Experimental design for the pathogenicity experiment

Room	Pen A	Pen B
1	25 SPF day-old chicks inoculated with sterile broth = (**L-NI)**	25 Ross 308 day-old chicks inoculated with sterile broth = **(R-NI)**
2	25 SPF day-old chicks inoculated with APEC 19-381 =(**L-381)**	25 Ross 308 day-old chicks inoculated with APEC 19-381 = (**R-381)**
3	25 SPF day-old chicks inoculated with APEC 19-383-M1 =(**L-383M)**	25 Ross 308 day-old chicks inoculated with APEC 19-383-M1 = (**R-383M)**
4	25 SPF day-old chicks inoculated with APEC 19-385-M1 = (**L-385M)**	25 Ross 308 day-old chicks inoculated with APEC 19-385-M1 = (**R-385M)**

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570 **Table 2.** Experimental design for the passive immunization experiment.

[a]Group (group size)	Group type	Chicks from vaccinated hen	*E. coli* challenge strain
NVNC (20)	Negative control	No	No
NVC381 (20)	Non-vaccinated and challenged 19-381	No	19-381
NVC383M (20)	Non-vaccinated and challenged 19-383-M1	No	19-383-M1
VNC (20)	Vaccine control	Yes	No
VC381 (20)	Vaccinated and challenged 19-381	Yes	19-381
VC383M (20)	Vaccinated and challenged 19-383-M1	Yes	19-383-M1

571 [a:] The vaccine for hens is a bivalent autogenous vaccine prepared with E. coli 19-381 and 19-383-M1 strains

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580 **Table 3.** Modes of the 39 significant variables for each cluster obtained using the k-means method performed on 268 *E. coli* strains and 45 descriptive

581 variables

Order of significance	Variable	Mode Cluster 1 (n=57)	Mode Cluster 2 (n=82)	Mode Cluster 3 (n=129)
1	Phylogroup	B2 (57)	F (74)	B1 (72)
2	Serogroup	O2 :K1(21)	O ?:H4 (52)	- (47)
3	*tkt1*	+ (57)	+ (82)	- (0)
4	*ibeA*	+ (55)	- (1)	- (0)
5	frz_{orf4}	+ (56)	+ (82)	- (6)
6	*pic*	- (1)	+ (72)	- (0)
7	*csgA2*	+ (57)	- (7)	- (4)
8	*aec4*	+ (29)	+ (82)	- (0)
9	*csgA1*	- (0)	+ (75)	+ (118)
10	*yqic*	+ (57)	+ (82)	- (27)
11	*ETT2.2*	- (1)	- (1)	+ (91)
12	*fepC*	- (17)	- (8)	+ (114)
13	*fimA1*	- (7)	+ (82)	- (50)
14	*hcp*	- (1)	- (1)	+ (81)
15	*fepA3*	+ (31)	+ (82)	+ (129)
16	*ireA*	- (11)	+ (79)	- (47)
17	*hra*	- (7)	- (3)	+ (78)
18	*fyuA*	+ (57)	+ (60)	- (41)
19	*fimA2*	+ (47)	+ (82)	- (57)
20	*pabB*	+ (37)	+ (82)	+ (128)
21	*clpv non sakai*	- (0)	- (0)	- (49)
22	*ompT1*	+ (57)	+ (82)	+ (80)
23	*aec35*	- (0)	- (0)	- (36)
24	*ecpD1*	+ (46)	+ (82)	+ (129)
25	*ecpA1*	+ (46)	+ (82)	+ (129)
26	*ecpA2*	+ (46)	+ (82)	+ (129)

27	*astA*	- (2)	- (8)	- (52)
28	*papg allele II*	- (1)	- (31)	- (13)
29	*tsh*	+ (45)	+ (82)	+ (82)
30	*vat*	+ (45)	+ (82)	+ (82)
31	*sat2*	+ (38)	+ (64)	- (50)
32	*tia*	- (1)	- (31)	- (17)
33	*papA*	- (1)	- (30)	- (18)
34	*iutA1*	+ (50)	+ (57)	+ (122)
35	*clbN*	- (6)	- (0)	- (0)
36	*cldB*	- (6)	- (0)	- (0)
37	*iha*	+ (29)	+ (57)	- (50)
38	*cma*	- (28)	- (39)	- (36)
39	*csgA3*	- (0)	- (0)	- (7)

582 For each marker and each cluster, the table gives the most frequent result (+ for presence or – for absence) and the number in brackets is the number of
583 isolates positive for the variable

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586 **Table 4.** Characteristics of the three challenge strains selected for use in the pathogenicity experiment

Strain (cluster)	Origin of isolation*	Serogroup phylogroup and ST**	Virulence-associated genes detected by PCR or WGS***	Antimicrobial Resistance genes
19-381 (cluster 1)	pericardium	O50/O2:K1 :H5 B2 ST140	*aec4, chuA, celB2, csgA2, cvaC, ecpD1, ecpA, etsC, fimA, fepA3, frzorf4, fyuA, hlyF, ibeA, ireA, iroN, irp2, iss, iucC, iutA, kpsE, mchF, neuC, nirC, ompT, pabB, phoB, rstA, sat2, sitA, terC, tkt1, traT, tsh, usp, vat, yfcv, YjjQ, YqiC*	*bla*$_{TEM-1B}$, *tet(A), sul1, sul2, dfrA1, strA, strB, aadA1*
19-383-M1 (cluster 2)	liver	O24:H4 F ST117	*aec4, cea, celB2, chuA, cma, csgA1,cvaC, ecpD1, ecpA1, ecpA2, fimA, fepA3, frzorf4, fyuA, hlyF, iha, ireA, iroN, irp2, iss, lpfA, mchC, mchF, neuC, nirC, ompT, pabB, phoB, pic, rstA, sat2, sitA, terC, tkt1, traT, tsh, vat, YjjQ, YqiC*	*bla*$_{TEM-1B}$, *tet(A), sul1, sul2, dfrA1, strB, strA, aadA1, mph(B), catA1*
19-385-M1 (cluster 3)	yolk	O86:H51 B1 ST155	*celB2, cia, cvaC, csgA1, ecpD1, ecpA1, ecpA2, etsC, ETT2,-fepC, fimA2, fepA3, hcp, hlyF, ireA, iroN, iss, lpfA, mchF, nirC, ompT, pabB, phoB, papA-papG allele II, rstA, sat2, sitA, terC, tia, traT, YjjQ*	*tet(B), sul2*

587 *origin of the strain or of its parental strain (organ from colibacillosis suffering chick); **serogroup, sequence type (ST) and resistance genes determined by

588 WGS and CGE server; ***detection by high-throughput PCR (Delannoy *et al.*, 2020) and/or virulence genes screened for using the CGE tool

589 Underlined genes were detected by PCR and WGS

Table 5. Pathogenicity experiment results: subcutaneous inoculation of three APEC strains (19-381, 19-383-M1 and 19-385-M1) to assess their virulence (mortality and clinical status) in day-old chicks for two chicken breeds (Ross 308 and Leghorn).

Challenge strain	Breed	Group	Mortality[a] (%)	Clinical status (x,y,z)[b]								
				D1	D2	D3	D4	D5	D6	D7	D8	D9
Negative control	Ross 308	R-NI	0^{A}	$0,0,0^{A}$ from D1 to D9								
	Leghorn	L-NI	0^{D}	$0,0,0^{C}$ from D1 to D9								
19-381	Ross 308	R-381	100^{C}	No	Surviving	Chicks	from	D1	to	D9		
	Leghorn	L-381	100^{F}	$6,0,0^{C}$	$1,3,1^{C}$	$0,0,1^{C}$	No	surviving	chicks	From	D4 to	D9
19-383-M1	Ross 308	R-383M	84^{B}	$13,3,0^{B}$	$7,7,0^{B}$	$4,8,1^{B}$	$4,3,3^{B}$	$3,1,2^{A}$	$4,0,0^{A}$	$4,0,0^{A}$	$4,0,0^{A}$	$4,0,0^{A}$
	Leghorn	L-383M	52^{E}	$19,1,0^{D}$	$16,3,1^{D}$	$15,5,1^{D}$	$13,3,1^{D}$	$11,2,3^{C}$	$12,1,0^{C}$	$12,0,0^{C}$	$12,0,0^{C}$	$12,0,0^{C}$
19-385-M1	Ross 308	R-385M	0^{A}	$0,0,0^{A}$ from D1 to D9								
	Leghorn	L-385M	0^{D}	$0,0,0^{C}$ from D1 to D9								

a comparisons were carried out within each chicken breed. In this column, percentages with different superscripts (A, B, C for the Ross 308 and D, E, F for Leghorn) are significantly different ($p<0.05$)

b (x,y,z) for each study day corresponds to the number of chicks that were scored 0, 1 or 2 (respectively normal, slightly depressed, prostrate). From D1 to D9, each inoculated breed was compared with the corresponding negative control group (A, B for the Ross 308 and C, D for Leghorn). For a given chicken breed, the clinical status score distributions with different superscripts are significantly different ($p<0.05$)

603

604 **Table 6.** Evaluation of the protection of day-old chicks against an *E. coli* homologous challenge via the passive immunization conferred by their autogenous
605 vaccinated mother hens. The autogenous vaccine, inactivated and adjuvanted, was prepared from *E. coli* strains 19-381 and 19-383-M1.

Group	Mortality[a] (%)	Clinical status[b]								
		D1	D2	D3	D4	D5	D6	D7	D8	D9
Negative control	0 [A]	0,0,0 from D1 to D9								
Non-vaccinated and challenged 19-381	90 [D]	4,13,0	3,1,0	3,1,0	3,0,1	3,0,0	3,0,0	3,0,0	3,0,0	3,0,0
Non-vaccinated and challenged 19-383-M1	80 [B]	7,13,0	5,0,0	4,0,0	4,0,0	4,0,0	4,0,0	4,0,0	4,0,0	4,0,0
Vaccine Control	0 [A]	0,0,0 from D1 to D9								
Vaccinated and challenged 19-381	95 [D]	2,15,1	1,1,0	1,1,0	1,1,0	1,0,0	No surviving chicks from D6 to D9			
Vaccinated and challenged 19-383-M1	40 [C]	16,4,0	15,0,0	14,0,0	12,0,0	12,0,0	12,0,0	12,0,0	12,0,0	12,0,0

606 a comparisons were carried out within each APEC strain (grew line for 19-381 and bold line for 19-383-M1). In this column, percentages in compared groups with different superscripts are significantly different ($p<0.05$)

607 b (x,y,z) for each study day corresponds to the number of chicks that were scored 0, 1 or 2 (respectively normal, slightly depressed, prostrate).

608

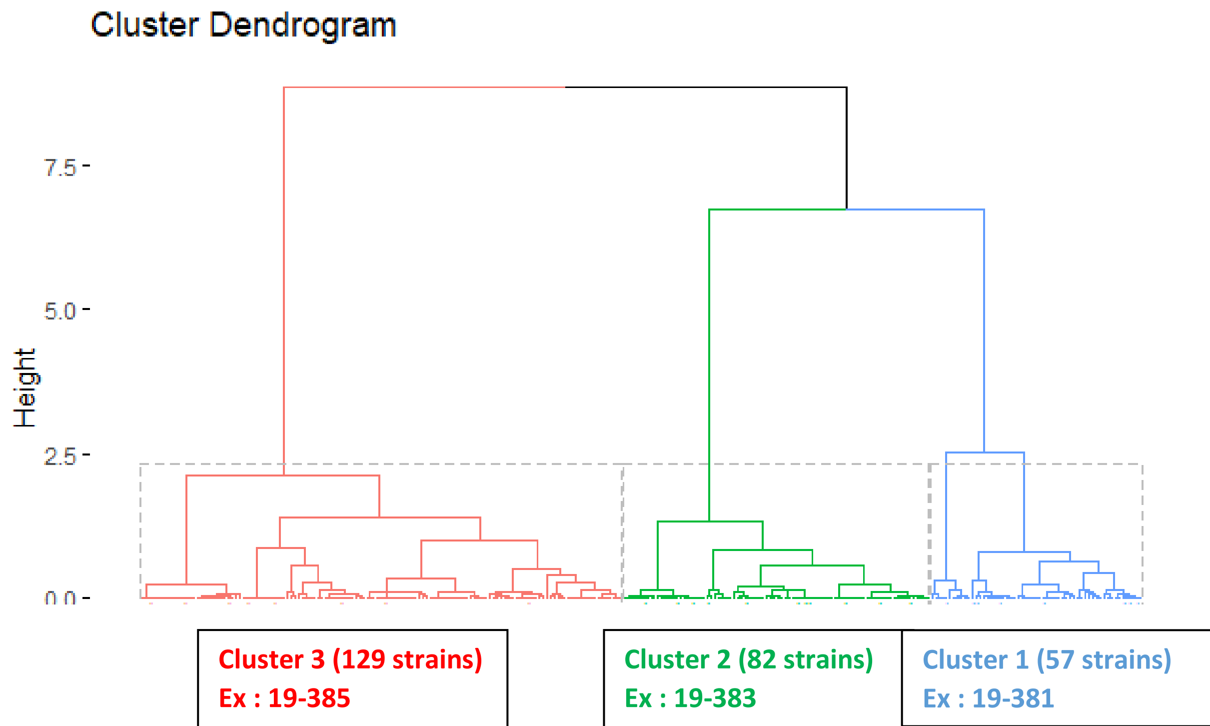

Figure 1: Dendrogram obtained by hierarchical clustering of 268 *E. coli* strains (x-labels) from 45 variables (i.e. phylogroup markers, serogroups and 66 virulence markers). These variables are used to calculate the distances between the 268 strains (y-labels).

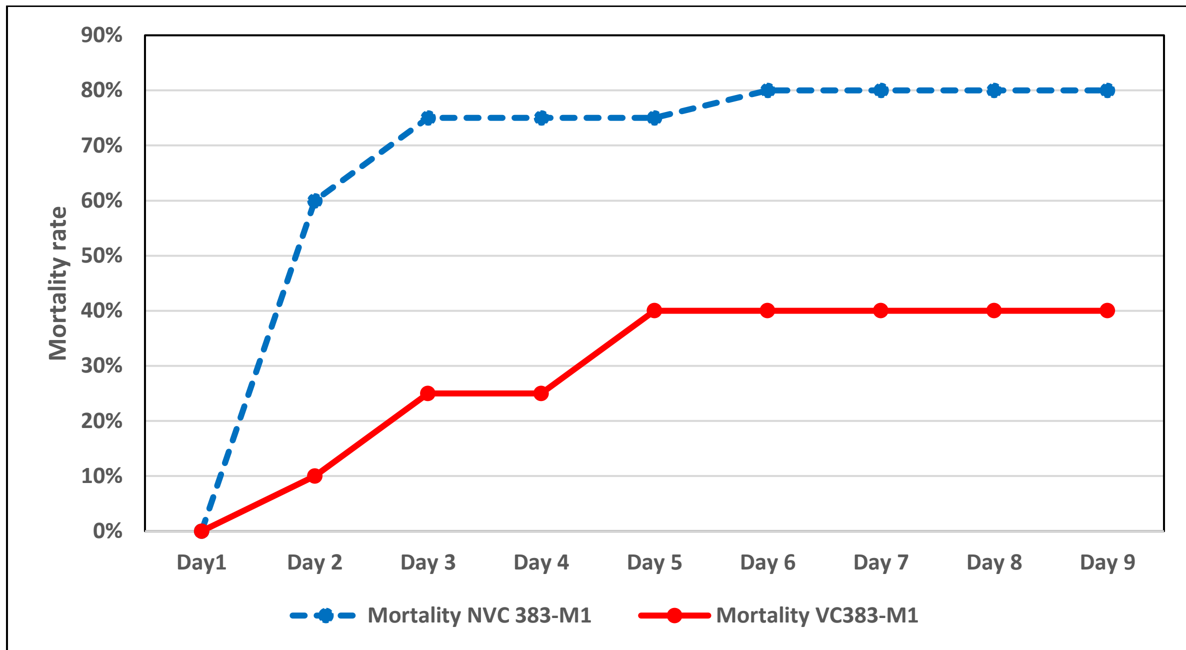

Figure 2: Mortality rate over time after challenge with the *E. coli* 19-383-M1 strain in non-vaccinated (NVC) and vaccinated (VC) chicks