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To cite this version:

Delphine Bibbal, Véronique Dupouy-Guiraute, Marie-Françoise Prère, Pierre-Louis Toutain, Alain Bousquet-mélou. Relatedness of Escherichia coli strains with different susceptibility phenotypes isolated from swine feces during ampicillin treatment.. Applied and Environmental Microbiology, 2009, 75 (10), pp.2999-3006. 10.1128/AEM.02143-08 . hal-00517533

HAL Id: hal-00517533

https://hal.science/hal-00517533v1

Submitted on 16 Sep 2010

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RELATEDNESS OF *ESCHERICHIA COLI* WITH DIFFERENT SUSCEPTIBILITY PHENOTYPES ISOLATED FROM SWINE FECES DURING AMPICILLIN TREATMENT

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Running title: Relatedness of fecal *Escherichia coli* isolates

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ABSTRACT

The aim of this study was to examine the dynamics of the development of resistance in fecal *Escherichia coli* populations during treatment with ampicillin for 7 days in pigs. Before treatment, only 6% of the isolates were ampicillin-resistant, whereas more than 90 % were resistant at days 4 and 7 of the treatment. Ampicillin-resistant *E. coli* isolates were mainly multi-resistant and one phenotype carrying resistance to six antibiotics (ampicillin, chloramphenicol, sulfonamides, tetracycline, trimethoprim and streptomycin) represented 53% of the isolates at day 7 in the treated pigs. A determination of the frequency of the four phylogenetic groups showed a shift in the *E. coli* population in ampicillin treated pigs: before treatment 75% of the isolates belonged to phylogroup B1, whereas at day 7, 85% of the isolates belonged to phylogroup A. PFGE (Pulsed-Field Gel Electrophoresis) typing revealed that ampicillin treatment selected ampicillin resistant isolates belonging to genotypes which were present before treatment. Comparison of antimicrobial phenotypes and PFGE genotypes showed that resistance traits were disseminated by vertical transmission through defined strains. One PFGE genotype, associated with the six-antibiotic-resistant phenotype, and harboring a specific combination of resistance determinants, was preponderant among ampicillin resistant strains before treatment and during treatment. These data indicate that ampicillin administration selected various ampicillin resistant isolates preexisting in the digestive tract before any treatment; and that *E. coli* isolates belonging to one PFGE genotype, carrying resistance to six antibiotics, became the predominant strains as soon as ampicillin was present in the digestive tract.

42 **INTRODUCTION**

43

44 Antimicrobial resistance in food animals deserves special attention, especially in pig
45 farming where worldwide consumption accounts for 60% of the antibiotics used in animals
46 (14). The digestive tract of pigs can harbor antimicrobial resistant bacteria among the
47 commensal flora, which form a reservoir of antibiotic resistance genes potentially
48 transmissible to humans via the food chain and the environment (36, 38, 45). *Escherichia coli*
49 is an indicator species for studies on the antibiotic resistance level of the fecal flora and has
50 been commonly used for this purpose in pigs (42). Epidemiological studies were performed
51 on commensal and pathogenic *E. coli* strains to identify the genetic determinants of the
52 resistance. They provided a descriptive and molecular epidemiology of fecal antimicrobial-
53 resistant bacteria from animals (4, 21, 26, 39). In addition, a relationship has been
54 demonstrated between the high use of antimicrobials in pig herds and the increased
55 occurrence of resistant bacteria strains in their digestive tracts (3, 17, 45). However, the
56 population processes underlying the emergence and spread of these antibiotic resistant strains
57 are little known and how these strains are selected by antibiotic administration in the gut
58 ecosystem remains unclear.

59 In an experimental setting, we previously showed that administration of ampicillin for
60 7 days in pigs led, whatever the mode of administration (intramuscular route, oral route in fed
61 or fasted conditions), to a large increase in ampicillin resistance in the fecal *E. coli* population
62 (2). Quantification of bla_{TEM} genes copies in swine feces by real time PCR also indicated an
63 increased excretion of these genes in treated animals and a significant correlation between the
64 quantities of bla_{TEM} genes and the counts of ampicillin-resistant *Enterobacteriaceae*,
65 confirming that these genes code for the most frequent mechanism of ampicillin resistance
66 among *Enterobacteriaceae* (28). As bla_{TEM} genes are plasmid-mediated, two mechanisms

67 could have contributed to the emergence of ampicillin resistance among fecal *E. coli*
68 populations during ampicillin selection pressure: whether ampicillin selected resistant strains
69 already present within the intestinal *E. coli* populations of these pigs, and/or previously
70 susceptible strains acquired bla_{TEM} genes by horizontal transfer. The aim of the present work
71 was to explore these mechanisms by studying the dynamics of resistance development in the
72 fecal populations of *E. coli* from pigs treated with ampicillin.

73 Our approach was therefore to investigate the relatedness of ampicillin resistant *E. coli*
74 in order to determine whether resistance emerged from genetically related or unrelated strains.
75 PFGE allows the identification of strains by their molecular genotype and has great value in
76 epidemiological analysis, in the differentiation of pathogenic strains, and in monitoring their
77 spread among communities (15, 18, 25). This method can also be used to evaluate the
78 potential clonal spread of resistant strains (1, 43). It has already been used to investigate the
79 dissemination and diversity of ampicillin-resistant commensal *E. coli* strains in cattle (23, 24)
80 and dogs (32).

81 In the present study, fecal *E. coli* isolated from pigs were characterized by their
82 antimicrobial resistance pattern, phylogenetic group and PFGE (Pulsed-Field Gel
83 Electrophoresis) genotype. This characterization performed before and during ampicillin
84 treatment enabled us to investigate whether resistance traits were disseminated through
85 specific strains or whether lateral spreading of bla_{TEM} genes to previously susceptible strains
86 occurred.

87 **MATERIALS AND METHODS**

88

89 **Study design and bacterial isolation.** Eighteen 7-week old, commercial healthy
90 piglets, that had never received antibiotics, were used. They were housed separately in
91 individual pens throughout all the experiments. Ampicillin was administered once a day at 20
92 mg/kg for seven days following three modalities: intramuscular route, oral route in fasted or
93 fed pigs. The design schedule has been detailed in our previous study (2). Briefly, it consisted
94 in three temporary independent experiments involving 6 pigs per experiment, which were
95 treated as follows: 4 pigs received ampicillin following 2 treatment modalities (2 pigs per
96 modality) and 2 pigs were used as control. Intramuscular injections of sodium ampicillin
97 (Ampicilline Cadril, Laboratory Coophavet, Ancenis, France) were administered in the neck.
98 For oral routes, a medicinal premix (Ampicilline 80 Porc Franvet, Laboratory Franvet, Segré,
99 France) was dissolved in water and administered by gastric intubation. All pigs came from the
100 same herd, which used a 3-week batch farrowing management. Pigs of one series came from
101 the same batch. All procedures involving animals were performed in accordance with the
102 French legal requirements regarding the protection of laboratory animals and with the
103 authorization for animal experimentation n° 31-242 from the French Ministry of Agriculture.

104 Fecal samples were taken from each pig before treatment and after 4 and 7 days of
105 treatment. Control pigs were sampled at the same times. Feces (5g) from each pig were
106 homogenized with 45 mL of peptone water, including 30% glycerol, with a BagMixer
107 (Interscience, St. Nom, France). Tenfold serial dilutions of the filtrate were prepared, and 100
108 µL samples of the dilutions were spread on MacConkey agar (AEB 151602; AES, Ker Lann,
109 France) at days 0 (Day 0), 4 (Day 4) and 7 (Day 7). In order to select ampicillin resistant
110 strains before treatment (Day 0 AmpR), fecal samples were also plated on MacConkey agar
111 containing 128 µg/mL of ampicillin at day 0 (the breakpoint value proposed by the CLSI is 32

112 µg/mL (11)). For each pig, five colonies were picked at random from the MacConkey plates
113 without ampicillin at days 0, 4 and 7 and from the ampicillin-containing plates at day 0. When
114 lactose-negative colonies were present, they were also picked and an attempt was made to
115 conserve the proportion of the two phenotypes lactose-negative and lactose-positive. All
116 isolates were confirmed to be *E. coli* by the API 20E *Enterobacteriaceae* identification
117 system (bioMérieux, Marcy l'Etoile, France).

118 **Antimicrobial susceptibility testing.** Antimicrobial susceptibility tests were
119 performed by a disk diffusion method according to the CLSI standards (9) on Mueller-Hinton
120 agar (Bio-Rad Laboratories). *Escherichia coli* ATCC 25922 was used as the control strain.
121 The 14 antibiotic disks (Bio-Rad Laboratories) used in this study were ampicillin (10 µg),
122 amoxicillin-clavulanic acid (20 + 10 µg), cephalothin (30 µg), ceftiofur (30 µg), streptomycin
123 (10µg), gentamicin (10 µg), kanamycin (30 µg), neomycin (30 µg), nalidixic acid (30 µg),
124 ciprofloxacin (5 µg), tetracycline (30 µg), chloramphenicol (30 µg), trimethoprim (5 µg) and
125 sulfonamides (300 µg). The susceptibility breakpoints for all antimicrobials were taken from
126 CLSI recommendations (10, 11), except for neomycin for which the breakpoints were
127 provided by the manufacturer.

128 **Pulsed field gel electrophoresis (PFGE)**. PFGE was performed using the method
129 described by Gautom with modifications (16). A bacterial suspension with A_{600} of 1.3 was
130 prepared with an overnight culture of the *E. coli* isolates in TE buffer (100 mM Tris and 100
131 mM EDTA, pH 7.5). Two hundred micro liters of the bacterial suspension were mixed with
132 10 µL of proteinase K (20mg/mL; MP Biomedicals, Qbiogene, France) and incubated for 15
133 min at 37°C. Twenty eight micro liters of 10% sodium dodecyl sulfate and 200 µL of 1%
134 Seakem Gold agarose (Lonza, Rockland, ME USA) prepared in SE buffer (EDTA 25 mM and
135 NaCl 75 mM, pH 7.5) were mixed with each bacterial suspension. This bacterium-agarose
136 mixture was added immediately to plug molds (Bio-Rad Laboratories, Hercule, CA, USA).

137 Solidified plugs were transferred to 1.5 mL ES buffer (0.5 M EDTA and 1% sodium lauryl
138 sarcosine, pH 9.0) and 40 µL of proteinase K (20mg/mL) and incubated for 1 hour at 55°C.
139 Plugs were then washed once with sterile distilled water and three times with TE buffer (10
140 mM Tris and 1 mM EDTA, pH 7.5) for 15 min each at 55°C. For restriction digestion, two 1
141 mm wide plug slices were incubated at 37°C for 3 hours with 30 U of *Xba*I enzyme (MP
142 Biomedical, Qbiogene, France) in the appropriate buffer. Plugs slices were loaded and
143 electrophoresed in 1% SeaKem Gold agarose with 2 L of 0.5 X TBE (Tris Borate EDTA).
144 Electrophoresis was performed with the Gene Path™ System (Bio-Rad Laboratories) using
145 the following conditions: initial switch time: 2.2 s, final switch time: 54.2 s, run time: 22 h,
146 angle 120°, gradient: 6 V/cm, temperature: 14°C, ramping factor: linear. The system was
147 manually shut down after 19 h of electrophoresis and the gels were stained in 500 mL distilled
148 water with ethidium bromide (Bio-Rad Laboratories) at the concentration of 0.5 µg/mL for 15
149 min followed by a one-hour wash with distilled water. A lambda ladder standard (Bio-Rad
150 Laboratories) was included in three lanes of every gel to allow fingerprint comparison
151 between different gels. Gel DNA bands patterns were analyzed using the Bio 1D++ software
152 (version 99, Vilber Lourmat). Fingerprints were clustered into groups by using the Dice
153 coefficient and evaluated by the unweighted-pair group method (1% position tolerance).
154 Clustered groups, in which more than two indistinguishable isolates were present, were
155 assigned a letter classification. A control strain belonging to PFGE group A was included in
156 each gel.

157 **PCR amplification.** DNA was extracted from all the *E. coli* isolates with the DNeasy
158 Blood and Tissue kit (Qiagen, Hilden, Germany) and stored at -20°C until required. The PCR
159 assays, using Taq DNA polymerase (MP Biomedicals, Qbiogene, France), were optimized
160 and performed using a PTC-200 thermocycler (MJ Research, USA). Phylogenetic lineage was
161 determined for all the *E. coli* isolates based on methods adapted from those of Clermont *et al.*

162 (8). Three separate PCR were run with primers targeting the genes *chuA*, *yjaA* and the
163 TspE4.C2 anonymous DNA locus. Samples which did not produce any positive amplicons
164 were amplified with primers targeting 16S rDNA genes from *Enterobacteriaceae* (6).
165 Representative ECOR (*E. coli* Reference Collection) strains were used as template controls.
166 The major resistance genes to beta-lactams (bla_{TEM}), sulfonamides (*sulI*, *sulII* and *sulIII*),
167 phenicols (*floR*, *cmlA*, *catI*, *catII* and *catIII*), streptomycin-spectinomycin (*strA-strB* and
168 *aadA1*), tetracycline (*tet*(A) and *tet*(B)) and the integrase genes *intI1* and *intI2* were detected
169 using the primers and the *E. coli* control strains listed in Table 1.

170 **RESULTS**

171 As no major differences were observed between the three treatment groups for the
172 distribution of antimicrobial resistance phenotypes, PFGE genotypes and phylogenetic
173 groups, the results from the ampicillin-treated pigs were pooled.

174 **Increase in resistance during ampicillin treatment.** Antimicrobial susceptibility
175 tests were performed on a total of 308 *E. coli* isolates. Table 2 shows the percentages of *E.*
176 *coli* isolates resistant to ampicillin, cephalothin, chloramphenicol, tetracycline, trimethoprim,
177 streptomycin, and sulfonamides for ampicillin-treated and control pigs at days 0, 4 and 7.
178 None of the *E. coli* isolates was resistant to ceftiofur. Three isolates were resistant to the
179 association amoxicillin-clavulanic acid and two isolates were resistant to gentamicin,
180 kanamycin, neomycin, ciprofloxacin and nalidixic acid. Before treatment, only 23% of the
181 *Escherichia coli* isolates were susceptible to all the tested antibiotics. Most of the isolates
182 were resistant to tetracycline (69%), and 46%, 45% and 20% were resistant to sulfonamides,
183 trimethoprim and streptomycin respectively. Only 6 % of the isolates were resistant to
184 ampicillin. In the ampicillin-treated group, the percentage of ampicillin resistant *E. coli*
185 isolates reached 93% at day 4 and 96% at day 7, and the percentages of resistance to other
186 antibiotics also increased. In contrast, in the control group, the percentages of isolates
187 resistant to ampicillin never exceeded 25%.

188 **Ampicillin resistant antimicrobial phenotypes.** Eighteen different ampicillin-
189 resistant phenotypes were identified. Ampicillin-resistant isolates were mainly multi-resistant
190 (resistant to ≥ 4 tested antimicrobial agents). They frequently carried additional resistance to
191 tetracycline, streptomycin, sulfonamides and trimethoprim. Table 3 shows the percentage of
192 the 6 main ampicillin-resistant phenotypes of *E. coli* isolates from ampicillin-treated pigs at
193 days 0, 4 and 7. The main ampicillin-resistant phenotype carried resistance to five additional
194 antibiotics: chloramphenicol, sulfonamides, tetracycline, trimethoprim and streptomycin.

195 Before treatment (Day 0), the baseline level of the six-antibiotic resistant phenotype was 5%,
196 and after 7 days of treatment, this phenotype reached 53% of all the isolates, and was present
197 in 10 of the 12 treated pigs at day 7. In addition, ampicillin-resistant phenotypes that appeared
198 during treatment were also found in *E. coli* picked from ampicillin-containing agar before
199 treatment (Day 0 AmpR). The phenotype carrying resistance to 6 antibiotics was already the
200 most frequent among the ampicillin-resistant strains before treatment.

201 **Genetic composition of *E. coli* populations.** To investigate the effect of ampicillin
202 administration on the genetic composition of *E. coli* populations, the phylogenetic group of *E.*
203 *coli* isolates was determined for ampicillin-treated and control pigs at days 0 (Day 0), 4 (Day
204 4) and 7 (Day 7), and for *E. coli* picked from ampicillin-containing plates before treatment
205 (Day 0 AmpR) (Fig. 1). Ten of the 308 typed *E. coli* isolates were not classified. Before
206 treatment (Day 0), the four phylogenetic groups were represented in the *E. coli* isolates picked
207 from ampicillin-free plates. The most abundant phylogenetic groups were B1 (75%) and A
208 (18%), whereas groups B2 (4%) and D (1%) were rare. The percentages of isolates belonging
209 to group B1 remained high in the control group at days 4 and 7, whereas it fell to 10% in the
210 treatment group. In *E. coli* strains isolated from ampicillin-treated pigs, group A became the
211 most abundant phylogenetic group: 85% at day 4 and 82% at day 7. Among the ampicillin-
212 resistant isolates present before treatment (Day 0 AmpR), 46% belonged to group A, 37% to
213 group B1 and 13% to group D. To conclude, at day 4 and 7 of the treatment, ampicillin led to
214 a shift in the genetic composition of the fecal *E. coli* population, with the selection of
215 ampicillin-resistant isolates mainly belonging to group A.

216 **Characteristics and distribution of the PFGE genotypes.** Among the 308 *E. coli*
217 isolates subjected to PFGE typing, 46 genotypes were identified, and two isolates could not be
218 typed by the PFGE method. Eighteen different PFGE groups containing more than two
219 indistinguishable isolates were identified and named with a letter from A to R. Ten PFGE

220 groups of two indistinguishable isolates were also identified. The isolates belonging to these
221 ten groups were pooled in a group named 'Pr' (n=20). In addition, 18 unique fingerprints
222 were identified, and they were pooled in a group identified by 'Uq' (n=18). Considerable
223 variations in banding profiles were observed between the PFGE genotypes (data not shown).
224 Each pig involved in the design harbored 3 to 11 different PFGE genotypes (a mean of 6.8).
225 Figure 2 shows the distribution of the PFGE groups in the three series of the experiment. The
226 results indicated that pigs coming from different series (different batches) shared common
227 PFGE genotypes. Figure 2 also presents the relation between antimicrobial susceptibility
228 phenotype and PFGE group. For the main PFGE groups, a strong association between a
229 genotype and an antimicrobial phenotype was observed, and ampicillin resistance was clearly
230 associated with specific PFGE groups.

231 Figure 3 shows the percentage of *E. coli* isolates belonging to each PFGE type at days
232 0, 4 and 7 of the ampicillin treatment. This figure also shows the percentage of genotypes for
233 *E. coli* isolates picked from ampicillin-containing agar before treatment (Day 0 AmpR).
234 Before ampicillin administration, the main PFGE genotype on ampicillin-free plates was
235 group G (22% of the isolates), followed by group M (15%) (Fig. 3 Day 0). Two main
236 genotypes A and K carried resistance to ampicillin. These two genotypes were the most
237 abundant among the ampicillin-resistant isolates selected on ampicillin-containing plates
238 before treatment (Fig. 3 Day 0 AmpR). At days 4 and 7, ampicillin treatment selected
239 resistant *E. coli* isolates which were already present in the digestive tract before treatment
240 (Fig. 3 Day 4 and Day 7). Moreover, ampicillin selected *E. coli* isolates belonging mainly to
241 PFGE group A as this represented 53% of the isolates at day 7. PFGE genotypes associated
242 with susceptibility to ampicillin before treatment were never observed associated with
243 resistance to ampicillin during treatment.

244 **PFGE genotypes and ampicillin-resistant phenotypes.** Comparison of phenotype
245 profiles with PFGE genotypes revealed that 23 genotypes were associated with ampicillin-
246 resistant phenotypes. Within- and between-group antimicrobial phenotype combinations were
247 examined. Table 4 shows the percentage of PFGE genotypes associated with a given
248 ampicillin-resistant phenotype for *E. coli* isolates from ampicillin-treated pigs. This table also
249 shows the distribution of PFGE genotypes at days 0, 4 and 7 and for *E. coli* isolates picked
250 from ampicillin-containing plates before treatment. It can be seen that one PFGE genotype
251 could be associated with variable resistance patterns and that identical resistance patterns
252 were recorded in different PFGE genotypes. However, each resistance pattern was mainly
253 associated with one PFGE group, and this association remained stable during treatment.
254 PFGE group A was associated with the phenotype resistant to six antibiotics. This antibiotic-
255 resistant phenotype was also associated with 6 other PFGE genotypes: groups J, K, H, O and
256 2 unique fingerprints. However, 76% of the isolates which carried resistance to six antibiotics
257 belonged to group A.

258 **Resistance determinants and integrase genes**. Resistance determinants and integrase
259 genes were amplified in the seven PFGE genotypes associated with the phenotype resistant to
260 6 antibiotics. Table 5 shows that different combinations of resistance genes existed for this
261 phenotype. In the case of ampicillin resistance, 49 of the 50 ampicillin-resistant isolates tested
262 harbored bla_{TEM} genes. Strains belonging to PFGE group A harbored the following specific
263 combination: bla_{TEM}, *catI*, *sulI*, *sulII*, *tet*(B), *strA-strB* and *intI1* (except for one isolate).
264 Another combination: bla_{TEM}, *cmlA*, *sulI*, *sulIII*, *tet*(A), *aadA*1 and *intI* was found for
265 genotypes J and K. Four other different and unique combinations were also identified.

266 **DISCUSSION**

267

268 In this study we showed that ampicillin administration in swine led to clonal selection
269 of ampicillin-resistant fecal *Escherichia coli* belonging to phylogenetic group A. These strains
270 were already present at low level before treatment in the digestive tract of the pigs. We also
271 showed that the principal ampicillin-resistant phenotype selected by ampicillin administration
272 carried resistance to six antibiotics (ampicillin, chloramphenicol, sulfonamide, tetracycline,
273 trimethoprim, streptomycin). Moreover, this phenotype was mainly linked to one PFGE
274 genotype characterized by a specific combination of resistance genes, and which was already
275 preponderant among the ampicillin-resistant strains harbored by the pig intestinal flora before
276 any antibiotic treatment.

277 The animals involved in the present investigations were used in a previous study,
278 during which we performed counts of ampicillin-resistant *Enterobacteriaceae* in the same
279 fecal samples (2). We previously observed that ampicillin-resistant *Enterobacteriaceae*
280 (mainly *E. coli* isolates) increased in number (2), which is in agreement with the present
281 observation that ampicillin treatment was associated with an increase in the percentage of
282 ampicillin-resistant *E. coli* isolates from 6% to 96%. Altogether, these results suggest that
283 ampicillin treatment actually selected ampicillin-resistant *E. coli* strains in the digestive tract.

284 Before treatment, the main phylogenetic group among *E. coli* commensal strains was
285 group B1, followed by group A. Groups B2 and D, which potentially include virulent extra-
286 intestinal strains (5, 29, 33) were rare. The predominance of group B1 has also been observed
287 in the fecal *E. coli* population of bovine origin (22, 49). In swine, it was shown that
288 commensal strains mostly belonged to groups A and B1 (7), with occasional predominance of
289 phylogroup A (40, 50). In our study, ampicillin treatment led to a shift in the composition of
290 the fecal *E. coli* population with the selection of ampicillin-resistant strains belonging to

291 phylogroup A. This result can be related to those of Walk *et al.* (49) who found an
292 overabundance of resistant phylogroup A strains on conventional dairy farms compared with
293 organic farms. They hypothesized that this difference in the genetic composition of the
294 resident *E. coli* flora might be linked to a higher use of ampicillin in conventional farms.

295 Ampicillin-resistant strains that appeared during treatment were of several
296 antimicrobial phenotypes, and mainly multi-resistant (resistant to at least 4 antimicrobial
297 agents). These phenotypes were already present in the digestive tract before treatment. The
298 main ampicillin-resistant phenotype carried resistance to six antibiotics and was already the
299 most abundant phenotype on ampicillin-containing plates before treatment. We used PFGE
300 typing to assess the potential clonal spread of ampicillin-resistant strains. The distribution of
301 PFGE genotypes of *E. coli* isolates at days 0, 4 and 7 of treatment, and for *E. coli* isolates
302 picked from ampicillin-containing plates before treatment, showed that ampicillin
303 administration selected ampicillin- resistant *E. coli* isolates which were already present in the
304 digestive tract before treatment. Comparison of antimicrobial phenotypes with PFGE groups
305 revealed that the PFGE genotypes could be associated with different antimicrobial phenotypes
306 and that antimicrobial phenotypes could be associated with different PFGE genotypes. These
307 results suggest that horizontal transfer or enrichment of resistance determinants within the
308 intestinal *E. coli* population can occur. As we have shown that ampicillin resistance was
309 mainly correlated with the presence of bla_{TEM} genes, which are plasmid-mediated (28), the
310 presence of many different PFGE genotypes in the ampicillin-resistant population is not
311 surprising. However, each ampicillin-resistant phenotype was mainly linked to one PFGE
312 genotype. This result suggests that resistance traits were disseminated by vertical transmission
313 through defined strains. When Mentula *et al.* studied the impact of ampicillin administration
314 on the fecal flora in dogs, they also found a considerable diversity in ampicillin-resistant
315 strains using PFGE typing (32). Their results indicated that the selection of genetically

316 heterogeneous resistant *E. coli* occurred rather than the selection of a single clone or
317 emergence of resistance within previously susceptible strains. In our study, we have shown
318 that, among the various ampicillin-resistant strains, *E. coli* isolates carrying resistance to six
319 antibiotics were preponderant and were mainly associated with one genotype. In addition, this
320 six-antibiotic-resistant phenotype was already the most abundant in the ampicillin resistant *E.*
321 *coli* population before treatment.

322 In the present study, we used pigs coming from the same herd but from distinct
323 batches, which were temporally distinct (from 6 to 9 weeks) and from different sows. Our
324 results indicated that these pigs shared *E. coli* isolates associated with the same genotypes.
325 Such a result suggests that a reservoir of antimicrobial resistant *E. coli* could exist in the
326 environment of the pig farm, as already shown in cattle and broilers (39, 41). As mentioned
327 by others, mechanisms unrelated to specific antimicrobial selection could be implicated in the
328 wide distribution and maintenance of such antimicrobial resistance genes. These mechanisms
329 include plasmid addiction, close linkage to other selectively advantageous genes (44), limited
330 metabolic burden associated with carriage of antimicrobial resistance genes (13, 34) and in
331 some instances, a secondary advantage can be conveyed by the antimicrobial resistance genes
332 in the absence of specific antimicrobial drug selection (12). In our study, we showed that
333 among the 7 PFGE genotypes associated with the six-antibiotic-resistant phenotype, the
334 PFGE genotype A harbored a specific combination of antimicrobial resistance determinants.
335 As suggested by Livermore (27), we can hypothesize that this genotype carrying resistance to
336 ampicillin was particularly well adapted because it may represent a combination between
337 mechanisms of resistance that confer a small fitness burden with a biologically fit host strain.

338 To conclude, ampicillin administration in pigs selected ampicillin-resistant strains
339 from a reservoir that already existed in the commensal flora before treatment. One genotype
340 carrying resistance to 6 antibiotics persisted in the digestive tract at a low level and became

341 the predominant strain as soon as ampicillin was present in the digestive tract. The
342 accumulation of resistance in this strain might reflect the degree of selection pressure and the
343 fitness of the strain. In order to prevent the emergence and spread of resistance, the
344 mechanisms by which the antimicrobial resistance genes are maintained in the digestive tract
345 need to be identified.

346 **ACKNOWLEDGMENTS**

347 This work was supported by the Conseil Régional de Midi-Pyrénées grant 03011987.

348 We thank N. Arpaillange and G. Faucon for technical support and A. Ferran for critical
349 reading of the manuscript. We are grateful to B. Doublet, A. Cloeckaert, M. Grape and M.
350 Sunde for providing the control strains.

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508
509

510 **FIGURE LEGENDS**

511

512 FIG. 1. Histogram plots of phylogenetic groups A (■), B1 (□), B2 (▥) and D (▨) for *E. coli*
513 isolates from ampicillin-treated and control pigs at days 0, 4 and 7. *E. coli* isolates were
514 picked from ampicillin-free plates (Day 0, Day 4, Day 7) or from ampicillin-containing plates
515 before treatment (Day 0 AmpR).

516

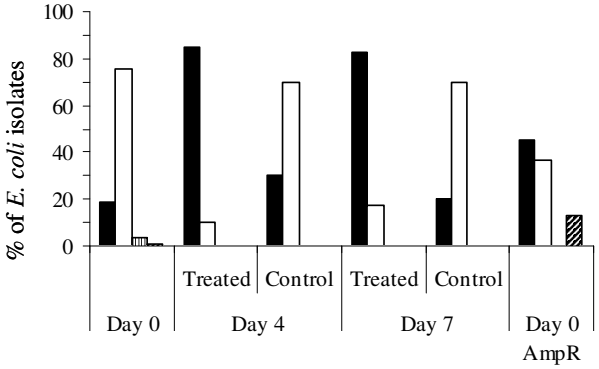
517 FIG. 2. Distribution of PFGE groups in the three series of the experiment and comparison of
518 antimicrobial susceptibility phenotypes versus PFGE group. The percentage of isolates
519 resistant to a given antimicrobial is indicated by block shading, with a grey box corresponding
520 to 100% and a white box to 0%. No: total number of isolates in the PGFE group; S.1, S.2 and
521 S.3: number of isolates in each time series; Amp, ampicillin; Chl, chloramphenicol; Sul,
522 sulfonamides; Te, tetracycline; Trim, trimethoprim; Str, Streptomycin; Sus, susceptible.

523

524 FIG. 3. Percentage of PFGE genotypes for *E. coli* isolates at days 0, 4 and 7 of ampicillin
525 treatment. *E. coli* isolates were picked from ampicillin-free plates (Day 0, Day 4, Day 7) or
526 from ampicillin-containing plates before treatment (Day 0 AmpR). 'A' to 'Q': PFGE groups
527 of at least three indistinguishable isolates; 'Pr': the pool of 10 groups of two isolates; 'Uq':
528 the pool of 18 unique isolates. Ampicillin-resistant isolates are indicated in black and
529 ampicillin-susceptible ones in white.

530

531 FIGURE 1



532

533

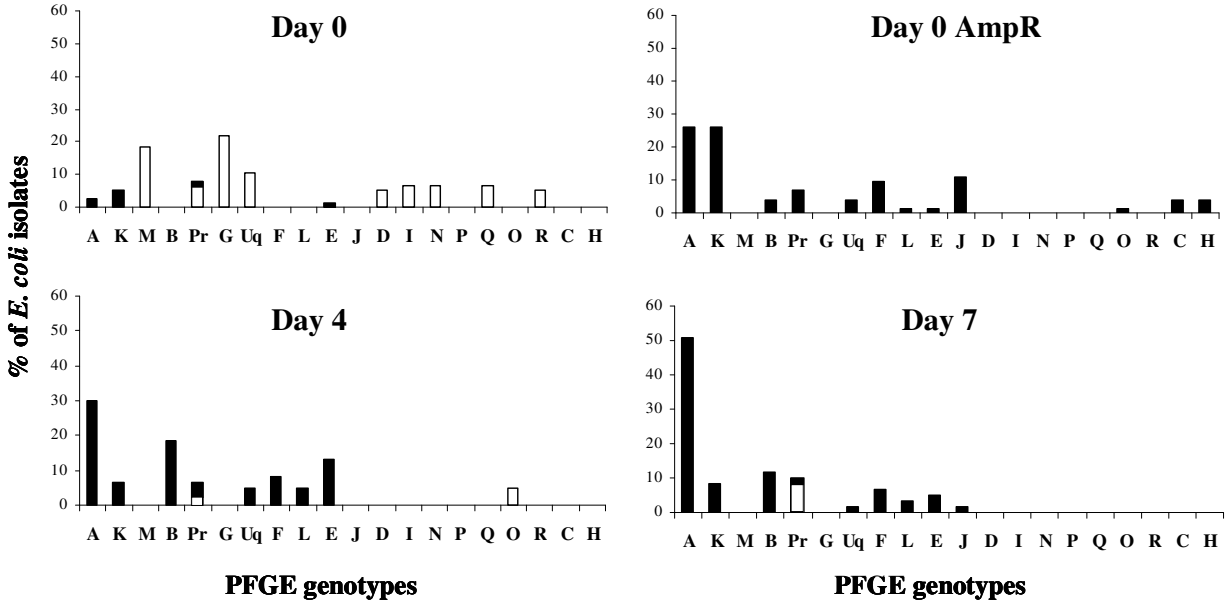
534 FIGURE 2

PFGE	No	S. 1	S. 2	S.3	Amp	Chl	Sul	Te	Trim	Str	Sus
A	69	21	17	31							
K	32	13	14	5							
M	32	5	26	1							
B	23		3	20							
G	18		5	13							
F	16	12		4							
L	15	1	2	12							
E	13	1	7	5							
J	9	3	5	1							
D	6			6							
I	5		5								
N	5	4	1								
P	5			5							
Q	5	4	1								
O	4		3	1							
R	4			5							
C	3	3									
H	3		3								

535

536

537 FIGURE 3



538

539

540 TABLE 1. PCR conditions and control strains

Gene	Primer sequence	Reference	Fragment size (bp)	Annealing (°C)	Positive control
bla$_{TEM}$	TTCCTGTTTTTGCTCACCCAG CTCAAGGATCTTACCGCTGTTG	2	112	60	JS238(pOFX326)[a]
sulI	TGGTGACGGTGTTCGGCATTC GCGAGGGTTTCCGAGAAGGTG	37	789	62	Se 131/ AJ238350[b]
sulII	CGGCATCGTCAACATAACC GTGTGCGGATGAAGTCAG	30	722	53	Se 678/ EF090911[b]
sulIII	CATTCTAGAAAACAGTCGTAGTTCG CATCTGCAGCTAACCTAGGGCTTTGGA	35	990	53	U39[b]
floR	CACGTTGAGCCTCTATAT ATGCAGAAGTAGAACGCG	37	868	55	BN10660[c]
cmlA	TGTCATTTACGGCATACTCG ATCAGGCATCCCATTCCCAT	37	455	57	1587 pcmlA[c]
catI	AGTTGCTCAATGTACCTATAACC TTGTAATTCATTAAGCATTCTGCC	30	547	53	BM14 R55 IncC[c]
catII	ACACTTTGCCCTTTATCGTC TGAAAGCCATCACATACTGC	30	543	50	BM21 Rsa IncW[c]
catIII	TTCGCCGTGAGCATTTTG TCGGATGATTATGGGCAAC	30	286	59	HB101 pUC CATIII[c]
strA-strB	TATCTGCGATTGGACCCTTGG CATTGCTCATCATTTGATCGGCT	46	538	60	Se 678/ EF090911[b]
aadA1	GAGAACATAGCGTTGCCTTGG TCGGCGCGATTTTGCCGGTTAC	46	198	53	Se 131/ AJ238350[b]
tet(A)	TTGTTCCTGAAGTGCCAGTAA GACGTCGTTCGAGTGAACCAGA	48	370	50	UA064 BM13/RP4[d]
tet(B)	CTCAGTATTCCAAGCCTTTG CTAAGCACTTGTCTCCTGTT	20	435	53	UA228 BM13/pIP69[d]
intI1	GGGTCAAGGATCTGGATTTCG ACATGGGTG TAAATCATCGTC	31	483	60	Se 131/ AJ238350[b]
intI2	CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	31	788	60	pR67[e]

541

542 [a] Reference 2

543 [b] Reference 47

544 [c] Provided by B. Doublet

545 [d] Provided by Institut Pasteur

546 [e] Reference 19

547

548 TABLE 2. Percentage of resistant *E. coli* isolates in ampicillin-treated and control groups at

549 days 0, 4 and 7

Antimicrobial	Day 0	Day 4 (n=80)		Day 7 (n=77)	
	(n=78)	Treated	Control	Treated	Control
Ampicillin	6	93	25	96	25
Cephalothin	0	3	5	7	0
Chloramphenicol	3	32	0	56	0
Tetracycline	69	90	85	89	65
Trimethoprim	45	58	0	68	5
Streptomycin	20	82	25	86	20
Sulfonamides	46	63	0	77	10
Susceptible	23	0	0	4	0

550

551 TABLE 3. Percentage of antimicrobial phenotypes for *E. coli* isolates from ampicillin-treated

552 pigs at days 0, 4 and 7. *E. coli* isolates were picked from ampicillin-free plates (Day 0, Day 4,

553 Day 7) or ampicillin-containing plates before treatment (Day 0 AmpR)

554

Antimicrobial phenotype[a]	Day 0 (n=58)	Day 4 (n=60)	Day 7 (n=57)	Day 0 AmpR (n=56)
Ampicillin susceptible phenotypes	88	6	5	0
Ampicillin resistant phenotypes	12	94	95	100
Amp Te		7	5	2
Amp Te Str		8	9	11
Amp Sul Te Str		15	9	2
Amp Chl Sul Trim Str	5	2	5	20
Amp Sul Te Trim Str		18	4	4
Amp Chl Sul Te Trim Str	5	32	53	48
Others	2	12	10	13

555 [a] Amp, ampicillin; Chl, chloramphenicol; Sul, sulfonamides; Te, tetracycline; Trim,

556 trimethoprim; Str, streptomycin

557 TABLE 4. Relation between PFGE genotypes and ampicillin-resistant phenotype for *E. coli*
558 isolates from ampicillin-treated pigs at days 0, 4 and 7. *E. coli* isolates were picked from
559 ampicillin-free plates (Day 0, Day 4, Day 7) or ampicillin-containing plates before treatment
560 (Day 0 AmpR).

Antimicrobial phenotype[a]	N[b]	PFGE genotype[c]				
		Day 0	Day 4	Day 7	Day 0 AmpR	Total
Amp Te	9		L (34) Uq (11)	L (22) A(11)	L (11) F (11)	L (67) Uq (11) A (11) F (11)
Amp Te Str	16		F (25) Pr (6)	F (25) Pr (6)	F (38)	F (88) Pr (12)
Amp Sul Te Str	20		B (50)	B (25)	B (15) Pr (10)	B (90) Pr (10)
Amp Chl Sul Trim Str	18	K (10) Pr (6)	K (6)	K (10) A (6)	K (44) A (6) Pr (6) Uq (6)	K (70) A (12) Pr (12) Uq (6)
Amp Sul Te Trim Str	18		E (40) A (6) B (10) K (6)	E (10)	E (6) A (6) H (10) Uq (6)	E (56) A (12) B (10) H (10) K (6) Uq (6)
Amp Chl Sul Te Trim Str	83	A (2) K (1)	A (20) Uq (2)	A (34) J (1) K (1)	A (20) J (10) K (7) H (1) O (1)	A (76) J (11) K (9) Uq (2) H (1) O (1)

561

562 [a] Amp, ampicillin; Chl, chloramphenicol; Sul, sulfonamides; Te, tetracycline; Trim,
563 trimethoprim, Str, streptomycin

564 [b] Number of *E. coli* isolates per phenotype

565 [c] 'A' to 'Q': PFGE groups of at least three indistinguishable isolates ; 'Pr': the pool of 10
566 groups of two isolates ; 'Uq': the pool of 18 unique isolates

567

568 TABLE 5. Distribution of antimicrobial resistance and integrase genes detected in the

569 genotypes associated with the phenotype profile Amp Chl Sul Te Trim Str[a]

PFGE type[b]	Phylo group	No[c]	Resistance genes detected
A	A	29	*bla*$_{TEM}$, *catI, sulI, sulII, tet*(B), *strA-strB, intI1*
A	B1	1	*bla*$_{TEM}$, *cmlA, sulI, sulIII, tet*(A), *aadA1, intI1*
J	B1	7	*bla*$_{TEM}$, *cmlA, sulI, sulIII, tet*(A), *aadA1, intI1*
J	A	1	*bla*$_{TEM}$, *cmlA, sulI, sulIII, tet*(A), *aadA1, intI1*
J	A	1	*bla*$_{TEM}$, *catI, sulI, sulII, tet*(B), *strA-strB, intI1*
K	B1	6	*bla*$_{TEM}$, *cmlA, sulI, sulIII, tet*(A), *aadA1, intI1*
K	A	1	*tet*(A), *strA-strB, aadA1, intI2*
O	B1	1	*bla*$_{TEM}$, *cmlA, sulI, sulIII, tet*(A), *aadA1, intI1*
H	D	1	*bla*$_{TEM}$, *sulI, sulII, tet*(A), *strA-strB, aadA1, intI1*
Uqa	B1	1	*bla*$_{TEM}$, *catI, sulI, sulII, tet*(A), *strA-strB, aadA1, intI1*
Uqb	A	1	*bla*$_{TEM}$, *tet*(A)

570

571 [a] Amp, ampicillin; Chl, chloramphenicol; Sul, sulfonamides; Te, tetracycline; Trim,

572 trimethoprim, Str, streptomycin

573 [b] Nominated groups are represented by the relevant letter, unique PFGE named x by 'Uqx'

574 [c] For a given genotype, all the isolates belonging to the genotype were screened except for

575 PFGE group A/ phylogroup A, for which 50% of the isolates were tested.

576

577

Point-by-point answers to reviewer's comments
AEM02143-08 Version 1

REVIEWER 1:

General comments

This study makes use of isolates from a previous investigation to assess the effect of experimental ampicillin treatment on antimicrobial resistance profiles and genotypes of E. coli in pigs. The results showed that specific ampicillin-resistant clones present before initiation of the ampicillin treatment were selected and replaced the susceptible strains in the treatment group. This study is very interesting and more investigations of this type are needed to understand better the in vivo population dynamics of bacteria under the selective pressure of antibiotics.

** The major weakness inherent to the original study design is the probable lack of complete replicates for the experiment, thus possibly reducing it to an experimental case study.*
Answer:

The results presented in this article came from an experiment performed on three successive series of pigs. The design of the experiment has been clarified in the Materials and Methods section, lines 93-103 of the corrected manuscript.

** In terms of molecular typing and antimicrobial resistance gene distributions, it would have been great to get some insights into what was happening not only in the treatment group over time, but also in the control group.*
Answer:

Some *E. coli* isolates from the control group were typed by PFGE at days 0, 4 and 7, and screened for resistance genes. The results indicated that as well as PFGE genotypes, genetic determinants of resistance at days 4 and 7 were similar to those detected at day 0. These results were not presented in the manuscript because we wanted to focus on the dynamics of resistance development during ampicillin treatment.

** Also, the use of a quantitative approach to the susceptibility testing (MICs) might be of interest in trying to explain further the selection of one major clone/resistance profile.*
Answer:

MICs for ampicillin had been previously determined in *E. coli* isolates originating from the same fecal samples used in the present study (Bibbal D. *et al.* **2007**. *Applied and Environmental Microbiology*, 73, 4785-4790), but not for the other tested antibiotics. We found that, as described in the literature (Livermore D. M. **1995**. *Clinical Microbiology Reviews*, 8, 557-584), ampicillin-sensitive isolates exhibited MICs of 2-8 µg/mL and that

(nearly) all ampicillin-resistant isolates had MICs equal to or above 256 µg/mL. Moreover, all tested isolates with MIC ≥ 256 µg/mL were positive for bla_{TEM} gene.

** Providing some statistical results on the comparisons between groups (using the animal as experimental unit and not the pen, to overcome at least partially the possible lack of replicates) would be welcome.*

Answer:

As discussed above, the experiment was performed on three series of pigs. We have modified Figure 2 in order to present the distribution of PFGE groups in the three series. We have chosen to describe results in a descriptive way, and we did not use statistical tools because a great number of PFGE genotypes (46 in total) and antimicrobial susceptibility profiles (18 ampicillin resistant phenotypes) were identified making the use of statistics inadequate compared with the number of *E. coli* isolates per pig and per day of sampling.

** Despite the description of the experimental design in a previous publication, it would certainly be nice to provide more details on this point in the present manuscript (see specific comments below).*

Answer:

We agree with the reviewer and we have clarified the experimental design in lines 89-117 in the corrected manuscript.

** The discussion is relatively confusing and essentially restricted to the repetition of the results section. It could be significantly shortened.*

Answer:

We have modified the discussion, taking into account the reviewer's comment. Repetitions of the results section have been deleted.

Specific comments

** Lines 86-88. What was the administration route for ampicillin and how does this dosage relate to doses commonly used in the practice of swine medicine?*

Answer:

Ampicillin was administered once a day at 20 mg/kg following three modalities: intramuscular route, oral route in fasted or fed pigs. This information has been added in lines 91-93 of the corrected manuscript. Ampicillin dosages were based on approved dosages for ampicillin in swine in France. For oral routes, the medicinal premix (Ampicilline 80 Porc Franvet, Laboratoire Franvet, Segré, France) is indicated at a dose of 20 mg/kg per day. The formulation for intramuscular route (Ampicilline 5G Cadril, Laboratoire Coophavet, Ancenis, France) is indicated with a dosage range of 14-28 mg/kg per day.

In the results section, we have chosen to pool the results from the ampicillin-treated pigs, whatever the mode of administration, because no major differences were observed between the three treatment groups for the distribution of antimicrobial resistance phenotypes, PFGE genotypes and phylogenetic groups. This information has been added in lines 171-173 of the corrected manuscript.

** Were replicates of the experiments done or is this the result of a single experiment? This is an important point in order to understand the real implications of the results.*

Answer:

The experimental design schedule consisted in three temporary independent experiments involving 4 to 6 pigs per experiment. All the pigs came from the same herd, which used a 3-weeks batch farrowing management. Pigs of one series came from the same batch and the time between the series was 6 weeks. The design schedule has been detailed in lines 93-103 of the corrected manuscript.

** Lines 95-103. What kind of filtration was used?*

Answer:

Feces from each pig and 45 mL of petone water, including 30% glycerol were placed in a BagFilter filter bag. These bags with integrated filters has been developed by Interscience in order to prepare filtrates for microbiological analysis. The bag was then placed in a BagMixer paddle blender (Interscience, St. Nom, France). In the filter bag, the sample was blended, homogenized and filtered thanks to the integrated filter of the bag.

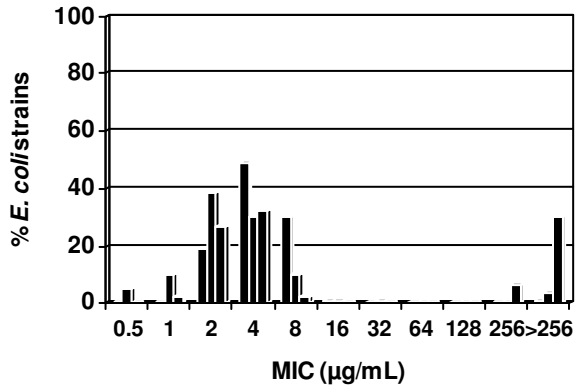
** Why was an ampicillin concentration four times higher than the breakpoint chosen for the selection of the resistant isolates? (were some susceptible isolates tested for the presence of beta-lactamase genes?)*

Answer:

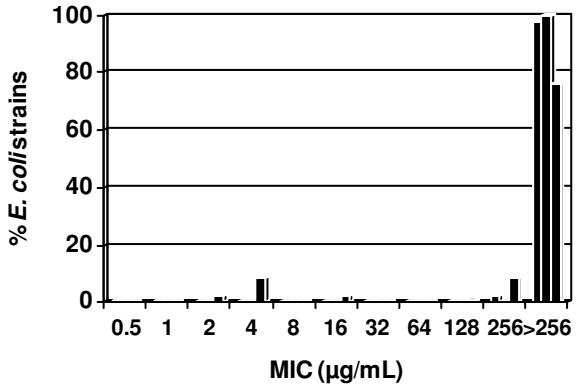
Please see the answer above and the following figure: nearly all ampicillin-resistant isolates had MICs above 128 µg/mL. Moreover, we screened some susceptible isolates for the presence of bla_{TEM} genes, and 2 out of 115 susceptible isolates were bla_{TEM} –positive. In addition, we showed in the previous study (Bibbal D. *et al.* **2007**. *Applied and Environmental Microbiology*, 73, 4785-4790) that a significant correlation existed between the quantities of bla_{TEM} genes in feces and the counts of ampicillin-resistant *Enterobacteriaceae*.

MICs for ampicillin of *E. coli* isolates before treatment (Day 0) and after 7 days of ampicillin treatment (Day 7):

Day 0



Day 7



** Please make clear in the text whether only lactose fermenters were picked on the MacConkey plates or if 'lactose-negative' E. coli strains were also considered.*

Answer:

We usually identified lactose positive strains (red colonies), associated with lactose negative isolates (white colonies) in some samples on MacConkey plates. When both phenotypes were visible, isolates were picked to conserve the proportion of the two phenotypes. This information has been added in lines 113-117 of the corrected manuscript. All isolates were then tested by the API 20E *Enterobacteriaceae* identification system (bioMérieux, Marcy l'Etoile, France) to confirm their identification. The results revealed that all tested strains were *E. coli*.

** Lines 108-111. Add streptomycin (and the breakpoint used for it) in this list since it appears later that it was also used for testing.*

Answer:

We have rectified this omission, lines 122 of the corrected manuscript. The breakpoint used for this antimicrobial was also taken from the CLSI recommendations.

** Line 207. What is meant by "ten pairs of isolates"? How does this differ from the indistinguishable isolates?*

Answer:

The following paragraph has been added in lines 216-222 of the corrected manuscript :

"Among the 308 *E. coli* isolates subjected to PFGE typing, 46 genotypes were identified, and two isolates could not be typed by the PFGE method. Eighteen different PFGE groups containing more than two indistinguishable isolates were identified and named with a letter from A to R. Ten PFGE groups of two indistinguishable isolates were also identified. The isolates belonging to these ten groups were pooled in a group named 'Pr' (n=20). In addition, 18 unique fingerprints were identified, and they were pooled in a group identified by 'Uq' (n=18)."

** Line 211 and lines 304-305. To address this properly, the authors should provide more information on the origin of the animals and how the experimental groups were assembled before discussing the point on similar clones originating from different herds.*

Answer:

As suggested by the reviewer, we have clarified the design schedule (please see above) and we have replaced the misused term "herd" by the appropriate term "batch". All the pigs came from the same herd, which used a 3-weeks batch farrowing management. As indicated above, pigs of one series came from the same batch and time between the series was 6 weeks. Moreover, we have presented the distribution of the PFGE groups in the three successive series (batches) in Figure 2.

** Table 3. Adding a row in the table indicating the total number of isolates resistant to ampicillin at each point in time would make the table more informative. Also, what are the denominators for these percentages (the column totals don't add up to 100%)?*

Answer:

Considering the reviewer's comment, we have modified the Table 3. The denominators for the percentages are the number of *E. coli* isolates at each sampling point.

** Tables 3 and 4. Since only ampicillin-resistant isolates are considered, it is not clear in the tables what the difference is between 'day 0' and 'day 0 AmpR'. Please clarify what the denominators are exactly.*

Answer:

Before treatment, *E. coli* isolates were plated on MacConkey plates:

- without ampicillin : “Day 0” and,
- with ampicillin : “Day 0 AmpR”.

Isolates obtained from ampicillin-free plates were tested for ampicillin resistance: a low baseline level of resistance was observed (6%). In order to characterize these strains better, we selected ampicillin-resistant isolates before treatment on ampicillin-containing plates (“Day 0 AmpR”). This information has been clarified in the Materials and Methods section, lines 107-111 of the corrected manuscript. Moreover, the following sentence has been added in the descriptions of Figure 1 and 3, and Tables 3 and 4: “*E. coli* isolates were picked from ampicillin-free plates (Day 0, Day 4, Day 7) or from ampicillin-containing plates before treatment (Day 0 AmpR)”.

** Table 4. Splitting the results to have only one PFGE type per line may make this table larger but also much easier to read.*

Answer:

We have modified this table, following the reviewer’s advice.

** Table 5. PFGE is a marvelous tool for epidemiology but known to be inadequate for phylogeny. However, the fact that an identical PFGE profile is found for isolates belonging to different phylogenetic groups on repeated occasions is striking (see type J and type K). The authors should comment on that point in the text.*

Answer:

We totally agree with the reviewer on this point. Nevertheless, the fact that an identical PFGE profile was found associated with different phylogenetic groups can be explained. PFGE typing involves digesting DNA with restriction endonucleases that cleave infrequently. We use the *Xba*I enzyme in our study, and DNA restriction patterns obtained depend on the number and the location of the restriction sites for this enzyme. Different PFGE banding patterns are the reflection of differences in the genome, but identical patterns are not synonymous with identical genomes. Concerning the identification of the phylogenetic group, it was based on the screening of 3 chromosomal genes by PCR according the method described by Clermont *et al.* (Clermont O. *et al.* **2000**. *Applied and Environmental Microbiology*, 66, 4555-4558). Strains belonging to different phylogenetic groups have at least one difference in the genome, but this difference is not necessarily seen when the strains are typed by PFGE. We can add this information in the text if the reviewer thinks it is necessary.

** Figure 2. I am not sure that this figure is really necessary and which message it is supposed to convey. A complete phenogram showing all the types isolated and the associated characteristics (i.e. susceptibility profiles, resistance genes detected) may be much more informative.*

Answer:

As suggested by the reviewer, we have deleted this figure and we have replaced it by a figure indicating the distribution of the PFGE groups in the three series and we have added information on the susceptibility profiles associated with the PFGE groups.

** Figure 3. The placement of the different parts of the figure is a bit unfortunate and does not facilitate the comparison between days 0, 4, and 7. Some other type of graphical presentation may make this comparison and the changes more obvious.*

Answer:

We have added information in the figure and in the text to clarify the comparison between days 0, 4 and 7.

REVIEWER 2:

This study examines the effect that ampicillin treatment in swine has on antibiotic resistance in E. coli as well as on the genetic makeup of the E. coli population before and after treatment. The data presented in this paper are important and are well-presented. Minor concerns with the manuscript are described below.

** The Introduction needs to be restructured. In the first paragraph, the authors use the past tense on lines 53-56, making it confusing to follow which studies are being referred to. The bigger problem is the second paragraph (there are only two in the entire Introduction) which starts with a justification of the study objective and hypothesis. It then drifts to a description of PFGE and finally wanders back to ampicillin administration and resistance. It might not be necessary to delete any of the information, but reorganization is suggested. In addition, an expansion of what the authors observed in their previous study would be useful. The one summary sentence on lines 62-64 does not do justice to the entire published study. It is the previous publication which justifies the hypothesis of this study*

Answer:

As suggested by the reviewer, we have modified and clarified the introduction.

** Lines 86-87: how was the antibiotic administered? Intramuscularly? Orally?*

Answer:

These informations have been added in the first paragraph of the Materials and Methods section (lines 92-93 of the corrected manuscript).

** More detail needs to be provided about the housing of the animals. Were the control animals housed with the treated animals?*

Answer:

Please see the response above.

** Line 88 states that the animals were handled in three successive series. What does this mean exactly? How many treatment and control animals were there in each series?*

Answer:

Please see the response above.

** What was the purpose of the 128 ug/ml ampicillin plates on day 0? Why were these only used on day 0? With a concentration that is two two-fold dilutions higher than the breakpoint, are the profiles observed representative of all ampicillin-resistant strains?*

Answer:

Isolates obtained in ampicillin-free plates were tested for ampicillin resistance: a low baseline level of resistance was observed (6%). In order to characterize these strains better, we selected ampicillin-resistant isolates before treatment on ampicillin-containing plates ("Day 0 AmpR"). This information has been clarified in lines 107-111 of the corrected manuscript.

MICs for ampicillin had previously been determined in *E. coli* isolates originating from the same fecal samples used in the present study (Bibbal D. *et al.* **2007**. *Applied and Environmental Microbiology*, 73, 4785-4790). We found that, as described in the literature (Livermore D. M. **1995**. *Clinical Microbiology Reviews*, 8, 557-584), ampicillin-sensitive isolates exhibited MICs of 2-8 µg/mL and that (nearly) all ampicillin-resistant isolates selected after treatment had MICs above 128 µg/mL.

** Perhaps the only criticism about the study design is the lack of enumeration data. The authors showed in their previous publication that blaTEM copy numbers were higher in the treated animals versus the control animals. This is potentially indicative of an actual increase in the population of bacteria harboring this gene, and consequently, evidence of selection. In this current study, is there a reason that the authors did not enumerate the E. coli population in the samples? Throughout the manuscript, the authors state that ampicillin selected for the ampicillin-resistant strains. Perhaps the ampicillin killed off the susceptible population and left the resistant population intact. Did the resistant population actually increase in number? Without this information, I am not sure that the authors can state that the treatment selected for resistance. I think they should rephrase the term "selection" throughout the manuscript to something like "ampicillin did not affect the resistant population."*

Answer:

The reviewer's comment is justified. In a previous study, we performed total counts of *Enterobacteriaceae* and ampicillin-resistant *Enterobacteriaceae* to calculate the percentages of resistant *Enterobacteriaceae* at each sampling point. Only these percentages were presented (Bibbal D. *et al.* **2007**. *Applied and Environmental Microbiology*, 73, 4785-4790).

In fact, the total counts of *Enterobacteriaceae* were stable in the ampicillin-treated pigs, and the counts of ampicillin-resistant *Enterobacteriaceae* largely increased. In that previous study, we also observed that the *Enterobacteriaceae* isolated in swine feces were mainly *E. coli* isolates. All the results indicated that ampicillin administration led to the selection of ampicillin-resistant *E. coli* strains. This point has been added to the discussion section lines 277-283.

** Figure 2 as presented does not seem to be useful. Detail could have been provided about the composition of each group. For example, line 304 states that pigs from different herds shared E. coli genotypes. No data are presented about inter-herd variability. This could have been included in the dendrogram. Days of isolation and treatment group could also have been included.*

Answer:

We have deleted this figure and we have replaced it by a figure indicating the distribution of the PFGE groups in the three series and we have added information on the susceptibility profiles associated with the PFGE groups.

** Table 3 is confusing. The numbers across the row or down the column do not sum to 100%. What is this table showing? Please clarify.*

Answer:

In the light of the reviewer's comment, we have modified Table 3. The denominators for the percentages are the number of *E. coli* isolates at each sampling point (each column).