



HAL
open science

Impact of three ampicillin dosage regimens on selection of ampicillin resistance in Enterobacteriaceae and excretion of blaTEM genes in swine feces.

Delphine Bibbal, Véronique Dupouy-Guiraute, Jean-Pierre Ferré, Pierre-Louis Toutain, Olivier Fayet, Marie-Françoise Prère, Alain Bousquet-mélou

► To cite this version:

Delphine Bibbal, Véronique Dupouy-Guiraute, Jean-Pierre Ferré, Pierre-Louis Toutain, Olivier Fayet, et al.. Impact of three ampicillin dosage regimens on selection of ampicillin resistance in Enterobacteriaceae and excretion of blaTEM genes in swine feces.. Applied and Environmental Microbiology, 2007, 73 (15), pp.4785-90. 10.1128/AEM.00252-07 . hal-00517560

HAL Id: hal-00517560

<https://hal.science/hal-00517560v1>

Submitted on 16 Sep 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Impact of three ampicillin dosage regimens on selection of ampicillin**
2 **resistance in *Enterobacteriaceae* and excretion of *bla*_{TEM} genes in swine feces**

3

4 D. Bibbal,¹ V. Dupouy,¹ J.P. Ferré,¹ P.L. Toutain,¹ O. Fayet,² M.F. Prère,² and A. Bousquet-
5 Mélou^{1*}

6

7 UMR181 Physiopathologie et Toxicologie Expérimentales, INRA, ENVT, Ecole Nationale
8 Vétérinaire de Toulouse, 23 Chemin des Capelles, BP 87 614, 31076 Toulouse Cedex 3,
9 France¹.

10 UMR5100 Microbiologie et Génétique Moléculaires, CNRS, UPS, Laboratoire de
11 Microbiologie et Génétique Moléculaires, Université Paul Sabatier, 118 route de Narbonne,
12 31062 Toulouse Cedex, France².

13

14

15 * Corresponding author. Mailing address: UMR181 Physiopathologie et Toxicologie
16 Expérimentales, INRA, ENVT, Ecole Nationale Vétérinaire de Toulouse, 23 Chemin des
17 Capelles, BP 87 614, 31076 Toulouse Cedex 3, France. Phone: 33 (0) 561 193 925. Fax: 33
18 (0) 561 193 917. E-mail: a.bousquet-melou@envt.fr

19 ABSTRACT

20 The aim of this study was to assess the impact of three ampicillin dosage regimens on
21 ampicillin resistance among *Enterobacteriaceae* recovered from swine feces using phenotypic
22 and genotypic approaches. Phenotypically, ampicillin resistance was determined from the
23 percentage of resistant *Enterobacteriaceae* and MICs of *E. coli* isolates. The pool of
24 ampicillin resistance genes was also monitored by quantification of *bla*_{TEM} genes, which code
25 for the most frequently produced β -lactamases in Gram-negative bacteria, using a newly-
26 developed real-time PCR assay. Ampicillin was administered intramuscularly and by oral
27 route to fed or fasted pigs for 7 days at 20 mg/kg. The average percentage of resistant
28 *Enterobacteriaceae* before treatment was between 2.5% and 12% and *bla*_{TEM} genes quantities
29 were below 10^7 copies/g of feces. By days four and seven, the percentage of resistant
30 *Enterobacteriaceae* exceeded 50% in all treated groups, with some highly resistant strains
31 (MIC>256 μ g/mL). In the control group, *bla*_{TEM} genes quantities fluctuated between 10^4 - 10^6
32 copies/g of feces, whereas they fluctuated between 10^6 - 10^8 and 10^7 - 10^9 copies/g of feces for
33 intramuscular and oral routes, respectively. Whereas phenotypic evaluations did not
34 discriminate between the three ampicillin dosage regimens, *bla*_{TEM} genes quantification was
35 able to differentiate between the effects of two routes of ampicillin administration. Our results
36 suggest that fecal *bla*_{TEM} genes quantification provides a sensitive tool to evaluate the impact
37 of ampicillin administration on the selection of ampicillin resistance in the digestive
38 microflora and its dissemination in the environment.

39 INTRODUCTION

40

41 The major mechanism of resistance to β -lactam antibiotics in Gram-negative bacteria
42 results from the production of β -lactamases. Most of these are coded by the plasmid-mediated
43 *bla*_{TEM-1} gene (16, 25). The continuous introduction of new β -lactam antibiotics with different
44 activity spectra in human medicine has led to the selection of β -lactamase mutations, which
45 confer resistance to the newly-developed β -lactam antibiotics (22). β -lactam antibiotics are
46 also used in veterinary medicine where they contribute to the selective pressure that leads to
47 the emergence and diffusion of intestinal bacteria harboring resistance genes. Thus,
48 commensal bacteria in the gut form a reservoir of antibiotic resistance genes potentially
49 transmissible to humans *via* the food-chain and the environment (24, 26, 30).

50 Antimicrobial resistance in food animals deserves special attention. One of the most
51 heavily medicated sectors is pig-farming, world-wide antibiotic consumption in pigs
52 accounting for 60% of the antibiotics used in animals (8). A relationship has been
53 demonstrated between the high use of antimicrobials in pig herds and increased occurrence of
54 resistant bacterial strains in their digestive tracts (4, 10, 30, 33). When antibiotics are
55 administered to pigs, both the level and time-development of antibiotic exposure of the
56 intestinal microflora are dependent on the mode of drug administration (34). This exposure is
57 a key determinant of antibiotic resistance development in the gut flora, and the relation
58 between antibiotic dosage regimen and resistance merits attention. The impact of different
59 antibiotic dosage regimens on the emergence of resistance must be evaluated by appropriate
60 quantitative indicators of the resistance level. Traditionally, this has involved phenotypic
61 methods that measure bacterial antibiotic susceptibility (28). In addition, quantitative PCR has
62 been recommended for resistance genes surveillance because i) it is sensitive ii) unambiguous

63 standard curves can be used to quantify the resistance genes from various matrices and iii) no
64 bacterial cultivation is required (12, 17, 27, 35).

65 The aim of the present study was both to develop and validate a real-time PCR assay
66 to quantify fecal *bla*_{TEM} genes in swine stools, and to explore the impact of three different
67 ampicillin dosage regimens on fecal ampicillin resistance in swine using different indicators.
68 Ampicillin resistance was evaluated by quantifying the *bla*_{TEM} genes in feces by real-time
69 PCR assay associated with two conventional phenotypic methods based on determination of
70 the MICs of *E. coli* isolates and the percentage of resistant *Enterobacteriaceae*. The three
71 dosage regimens tested were: intramuscular route, oral route in fed and oral route in fasted
72 swine.

73

74 MATERIALS AND METHODS

75 **Study design and sample collection.** Eighteen 7-week old, commercial healthy
76 piglets, that had never received antibiotics, were used. They were housed separately in
77 individual pens throughout all the experiments. A meal was given twice daily and water was
78 provided *ad libitum*. Ampicillin was administered once a day at 20 mg/kg for seven days
79 (from day 0 to day 6) following three modalities: intramuscular route, oral route in fasted pigs
80 or oral route in fed pigs. The design schedule consisted of three successive series of 6 animals
81 receiving ampicillin treatments as follows: intramuscular (n=2), oral route in fed conditions
82 (n=2), control without treatment (n=2) in the first series; intramuscular (n=2), oral route in
83 fasted conditions (n=2), control without treatment (n=2) in the second series; oral route in fed
84 conditions (n=2), oral route in fasted conditions (n=2), control without treatment (n=2) in the
85 third series. Six pigs were used in the control group and 4 pigs in each ampicillin treatment
86 group. Intramuscular injections of ampicillin sodium (Ampicilline Cadril, Laboratory
87 Coophavet, Ancenis, France) were administered in the neck. For oral routes, a medicinal
88 premix (Ampicilline 80 Porc Franvet, Laboratory Franvet, Segré, France) was dissolved in
89 water and administered by gastric intubation. Fasted swine were starved 16 hours before
90 ampicillin administration and fed 4 hours after ampicillin administration. Ampicillin was
91 administered to fed pigs just at the end of their morning meal.

92 For phenotypic evaluation of ampicillin resistance, fecal samples were taken from
93 each pig, by digital manipulation or immediately after spontaneous defecation, at days 0
94 (before ampicillin administration), 1, 4, and 7. The samples were immediately transferred to
95 the laboratory and the *Enterobacteriaceae* were counted. For the quantification of *bla*_{TEM}
96 genes in feces by real-time PCR, feces of each pig were collected two or three times before
97 the treatment. The value given for day 0 is the mean of these samplings. Feces were then
98 collected each day from day 1 to day 7. Samples were obtained as already described. Two

99 hundred mg of feces from each sample were frozen in liquid nitrogen and stored at -80°C
100 until assayed.

101 **Phenotypic evaluation of ampicillin resistance.** Feces (5 g) from each pig were
102 homogenized with 45 mL of peptone water, including 30% of glycerol, with a BagMixer
103 (Interscience, St Nom, France). Ten-fold serial dilutions of the filtrate were prepared and 100
104 µL of the dilutions were spread on MacConkey plates (AEB 151602, AES, Ker Lann, France)
105 containing 0 and 16 µg/mL of ampicillin. *Enterobacteriaceae* growing in the presence of 16
106 µg/mL of ampicillin were classified as resistant, considering the MIC breakpoint proposed by
107 the CLSI (MIC \geq 32 µg/mL) (20). The plates were incubated at 37°C for 24 h.
108 *Enterobacteriaceae* counts from both plates were used to calculate the percentage of resistant
109 *Enterobacteriaceae* at each sampling time.

110 For each sample, 20 colonies were randomly picked on the MacConkey plates without
111 ampicillin and stored at -80°C until assayed. These colonies were considered as *E. coli* on the
112 basis of β -glucuronidase production using TBX agar (Tryptone Bile X-glucuronide agar, AES
113 laboratoire, Bruz, France) (11). Only a few colonies were β -glucuronidase negative. All β -
114 glucuronidase negative isolates and a portion of β -glucuronidase positive isolates were tested
115 by the API 20E *Enterobacteriaceae* identification system (bioMérieux, Marcy l'Etoile,
116 France) to confirm their identification. For MICs determination, ampicillin susceptibility was
117 tested by microdilution broth dilution method according to the recommendations reported by
118 the CLSI (19). The control strain was *E. coli* ATCC 25922.

119 **Bacteria and growth conditions.** *E. coli* JS238[pOFX326], the plasmid of which
120 carries a monocopy of the target gene *bla*_{TEM-1}, was used to optimize real-time PCR, assess
121 sensitivity and generate quantification standards. The strain was cultured in Mueller-Hinton
122 broth containing ampicillin at the concentration of 50 µg/mL at 37°C overnight.

123 **DNA extraction.** pOFX326 was purified with the QIAprep Spin Miniprep (Qiagen,
124 Hilden, Germany). Quality was assessed by migration on gel electrophoresis in 1% agarose,
125 after digestion with *Hind*III and concentration was assessed by spectrophotometry at 260 nm.
126 The QIAamp DNA Stool kit (Qiagen, Hilden, Germany) was used to extract DNA from feces
127 according to manufacturer's recommendations. For each series of extractions, a positive
128 control and a negative control were co-extracted and subjected to real-time PCR.

129 **Design of primers.** The PCR primers were designed with Primer 3 and Oligo
130 Analyser. The specificity of the sequence was further checked against all the available
131 GenBank DNA sequences. The forward and reverse primers chosen for *bla*_{TEM} genes
132 quantification were 5'-TTCCTGTTTTTGCTCACCCAG-3' and 5'-
133 CTCAAGGATCTTACCGCTGTTG-3', respectively. These primers amplify a 112 bp
134 segment of the *bla*_{TEM-ID} gene (GeneBank accession number AF 1888200) from nucleotide
135 positions 270 to 382. A 100% homology was demonstrated with 130 *bla*_{TEM} genes for which
136 the nucleotide sequence was available, except for TEM-60.

137 **Real-time PCR assay.** The PCR amplification was performed in a 25 µL reaction
138 mixture with a SYBR Green PCR Core Reagents kit (Perkin Elmer Biosystems, Foster City,
139 USA). The reaction mixture contained 5 µL of test DNA solution, 2.5 µL of 10X SYBR
140 Green PCR Buffer, 1.6 µL of a deoxynucleoside triphosphate solution (2.5 mM each of
141 dATP, dCTP and dGTP and 5 mM of dUTP), 0.25 µL of each primer (20 µM), 4 µL of 25
142 mM MgCl₂, 11.275 µL of Ultra Pure Water (Qbiogene, Montréal, Canada) and 0.125 µL of
143 AmpliTaq Gold® DNA Polymerase, LD (5 U/µL) (Perkin Elmer Biosystems). Amplification
144 was performed using a GeneAmp® PCR System 5700 thermocycler (Perkin Elmer
145 Biosystems) with the following conditions: 95°C for 10 min followed by 45 cycles of 15
146 seconds at 95°C and 1 minute at 60°C. A standard curve with three replicates of the control
147 plasmid pOFX326 diluted in Tris-EDTA buffer was generated for each PCR assay. All

148 sample PCRs were done in duplicate. The samples were checked for absence of background
149 levels of PCR-inhibiting compounds by spiking DNA extracted from the samples with target
150 DNA and subjecting these spiked DNA samples to real-time PCR both undiluted and diluted
151 (1:10).

152 The impact of DNA fecal environment on amplification sensitivity and performance was
153 assessed by comparing standard curves obtained with the control plasmid diluted in Tris-
154 EDTA or in swine fecal DNA. The accuracy and reproducibility of the entire assay (from
155 DNA extraction to real time PCR analysis) was measured by spiking 200 mg of feces with an
156 overnight culture of *E. coli* JS238[pOFX326]. Five aliquots per day were subjected to DNA
157 extraction on three different days. The extraction recovery rate was calculated. It was checked
158 to be the same for different concentrations of *bla*_{TEM} genes in feces by spiking fecal samples
159 with 10-fold serial dilutions of an overnight culture of *E. coli* JS238[pOFX326]. These
160 samples were subjected to DNA extraction and then to real-time PCR.

161 **Statistical analysis.** Statistical analysis was performed using Systat 10 (Systat
162 Software Inc., Richmond, CA, USA). Changes in the level of ampicillin resistance were
163 analyzed using a generalized linear mixed-effects model with the following equation:

$$164 \quad Y_{ijk} = \mu + M_i + D_j + A_k|_{M_i} + M^*D_{ij} + \varepsilon_{ijk},$$

165 where Y_{ijk} is the measure of resistance for pig k undergoing ampicillin administration with
166 modality i at day j , μ the overall mean, M_i the differential effect of treatment i , D_j the
167 differential effect of day j , M^*D_{ij} the corresponding interaction, $A_k|_{M_i}$ the differential effect
168 of animal k nested within treatment i and ε_{ijk} an error term. Y , the measure of resistance, was
169 monitored in various ways. For the phenotypic evaluation of resistance, Y was the log-
170 transformed percentage of the resistant *Enterobacteriaceae* population or the log-transformed
171 percentage of *E. coli* isolates with MIC > 16 $\mu\text{g/ml}$. For the genotypic evaluation, Y was the

172 log-transformed quantity of *bla*_{TEM} genes. Multiple comparisons were performed using the
173 Tukey test. The selected level of significance was $P < 0.05$.

174 RESULTS

175

176 **Validation of the PCR assay.** In order to construct calibration curves and determine
177 the specificity and sensitivity of the primers in swine fecal DNA, the control plasmid
178 pOFX326 was diluted in Tris-EDTA buffer and in swine fecal DNA. Each dilution was
179 subjected to real time PCR and the amplifications were repeated four times. Melting-curve
180 analysis of the control plasmid, diluted either in Tris-EDTA buffer or in swine fecal DNA,
181 showed specific amplification with a PCR amplicon at a T_m value of 81°C (data not shown).
182 Despite the use of highly purified AmpliTaq Gold® DNA Polymerase, analysis of the Ultra-
183 Pure Water melting-curves revealed contamination and thus restricted the PCR quantification
184 limit (data not shown). Fig. 1 shows the two standard curves: the relation between Ct
185 (threshold cycles) values and the logarithm of bla_{TEM} concentration was linear from 10 to 10^6
186 copies/ μ L. The determination coefficients (r^2) were of 0.996 in Tris-EDTA and 0.985 in
187 swine fecal DNA. The closeness between these standard curves indicated that the complex
188 fecal DNA environment did not affect amplification sensitivity or performance. The intra- and
189 inter-day coefficients of variation of the entire assay (from DNA extraction to real time PCR
190 analysis) were 16.7% and 18.2%, respectively. The extraction recovery rate was 70-113%
191 (mean 98.5 %). This was checked to be the same for different concentrations of bla_{TEM} genes
192 in feces by spiking fecal samples with 10-fold serial dilutions of an overnight culture of *E.*
193 *coli* JS238[pOFX326]. The correlation between bla_{TEM} copy number/g feces and dilution
194 factors of the JS238[pOFX326] solution was high (with a determination coefficient,
195 $r^2=0.904$). Thus the extraction yields for different concentrations of *E. Coli* JS238[pOFX326]
196 in feces were similar. Overall data demonstrated that this PCR analysis was suitable for
197 quantification of bla_{TEM} genes in swine feces from 10 to 10^6 copies/ μ L of eluate of extracted
198 DNA, which corresponds to 10^4 to 10^9 copies/g of feces.

199 **Phenotypic evaluation of ampicillin resistance.** Average percentages of ampicillin-
200 resistant *Enterobacteriaceae* for each treatment group are given in Fig. 2a. The average
201 percentage of resistant *Enterobacteriaceae* ranged from 0.9% to 12% before ampicillin
202 administration. On the first day of treatment, it rose to 26% for the intramuscular route and to
203 40% and 49% for the oral routes in fed and fasted pigs respectively. By days 4 and 7, the level
204 of resistance exceeded 50% in all treated groups. In contrast, the level of resistance in the
205 control group remained below 13% at all times. Treated animals excreted significantly higher
206 percentages of resistant *Enterobacteriaceae* compared to the control group ($P<0.05$).
207 However, no significant differences were observed between the three modes of drug
208 administration ($P>0.05$). Furthermore, Fig. 2a shows the high inter-individual variability
209 within each group.

210 Ampicillin resistance was also monitored from the percentage of resistant *E. coli*
211 isolates for each treatment group (Fig. 2b). The average percentage of resistant *E. coli* ranged
212 from 1% to 38% before ampicillin administration. At day 1 of treatment, about 70% of
213 isolates were resistant, whatever the mode of drug administration. By days 4 and 7, nearly all
214 the isolates, whatever the dosage regimen, were resistant. In contrast, the percentages of
215 resistant *E. coli* remained below 36% in the control group. Statistical analysis indicated that
216 oral administration in fed pigs led to a higher fecal excretion of resistant *E. coli* than in
217 control pigs ($P<0.05$). The two other dosage regimens did not differ significantly from the
218 control group due to the great heterogeneity of the control group data ($P>0.05$). High inter-
219 individual variability also existed within each ampicillin-treated group.

220 **Genotypic evaluation of ampicillin resistance.** Ampicillin resistance in feces was
221 measured by *bla*_{TEM} genes quantification using the validated PCR assay. *bla*_{TEM} genes copy
222 numbers per gram of wet feces were measured on each day of treatment for each pig (Fig. 3).
223 The baseline values for all pigs were below 10^7 copies/g of feces. *bla*_{TEM} quantities increased

224 after ampicillin administration. The between-day fluctuations for a given animal were large.
225 The *bla*_{TEM} quantities for the oral routes fluctuated between 10⁷ and 10⁹ copies/g of feces, but
226 only between 10⁵ and 10⁸ copies/g of feces for the intramuscular route. Two fed pigs treated
227 by oral route excreted the highest *bla*_{TEM} quantities with values above 10⁹ copies/g of feces.
228 The *bla*_{TEM} quantities for the control group were lower than those of the three ampicillin-
229 treated groups and fluctuated between 10⁴ and 10⁶ copies/g of feces.

230 Fig. 4 shows the mean quantities of *bla*_{TEM} genes for each dosage regimen. Statistical
231 analysis indicated that all ampicillin treatments had a significant effect on the excretion of
232 *bla*_{TEM} genes compared to the control group ($P < 0.001$). Moreover, oral administration in fed
233 pigs led to a significantly higher excretion of *bla*_{TEM} genes than intramuscular administration
234 ($P < 0.05$).

235 **Comparisons of real time PCR assessments and phenotypic plate assays.** We
236 investigated the agreement between resistant *Enterobacteriaceae* counts and *bla*_{TEM}
237 concentrations. Fig. 5 shows a significant correlation (with a determination coefficient,
238 $r^2 = 0.67$) between the quantities of *bla*_{TEM} genes and the counts of ampicillin-resistant
239 *Enterobacteriaceae*.

240

241 **DISCUSSION**

242 The aim of this study was to explore the impact of three ampicillin dosage regimens
243 on the selection of ampicillin resistance in swine feces. Three indicators of ampicillin
244 resistance *i.e.* two classical phenotypic methods and a new genotypic method allowing
245 quantification of *bla*_{TEM} genes in feces were selected. The results, whichever resistance
246 indicator was used, indicated that the different modes of ampicillin administration led
247 immediately (day one of treatment) to a large increase in the level of ampicillin resistance in
248 the fecal microflora. In addition, the results suggested that the quantitative PCR of fecal
249 *bla*_{TEM} genes might be a promising tool to quantify the digestive reservoir of *bla*_{TEM} genes and
250 evaluate the impact of β -lactam administration on the selection of ampicillin resistance in the
251 gut microflora.

252 Antibiotic impact on the gut microflora is generally measured by phenotypic
253 evaluation of antibiotic resistance on a limited bacterial population, either using isolates of
254 indicator bacteria or families of bacteria. *E. coli* and *Enterobacteriaceae* are good candidates
255 for studies of the antibiotic resistance level of the fecal flora and are commonly used for this
256 in pigs (28). These bacteria are easily culturable and their isolation is facilitated by specific
257 culture media. In the present experiment, results obtained with the two phenotypic indicators
258 of ampicillin resistance implied that all treatments had a similar negative impact on the gut
259 microflora with the emergence of a high level of resistance at all three dosage regimens.
260 These results are consistent with those of previous studies demonstrating that ampicillin
261 treatment could have a marked effect on the level of resistance in intestinal microbiota of
262 several species (7, 18, 29). Nevertheless, the phenotypic indicators commonly used to assess
263 antibiotic resistance exhibit methodological features that impact both their metrological
264 performances and relevance. Firstly, the selected indicator bacteria must be cultured and the
265 reliability of results has been questioned due to considerable variation originating from the

266 culture medium, bacterial inoculum, antibiotic preparation and incubation conditions (23).
267 Secondly, the isolates might not be representative of the whole population of bacteria (6).
268 These limits impair the sensitivity and precision of phenotypic indicators for the assessment
269 of resistance levels and have prompted investigators to develop molecular techniques as
270 alternatives, in particular quantitative PCR (12, 17, 27, 35).

271 Molecular techniques can be used to reveal the presence of genetic determinants without
272 bacterial cultivation and irrespective of the bacterial species carrying these genetic
273 determinants (5, 31). However, a requisite to this approach is the knowledge of the underlying
274 resistance mechanisms, and when few genes are involved in resistance, they may provide
275 candidates for resistance markers (3). *bla*_{TEM} genes code for the most commonly encountered
276 β -lactamases in Gram-negative bacteria (21). We therefore developed and validated a real-
277 time PCR assay to quantify *bla*_{TEM} genes in swine feces. This PCR assay was suitable for
278 quantification of *bla*_{TEM} genes from 10^4 to 10^9 copies/g of feces.

279 Examination of the agreement between resistant *Enterobacteriaceae* counts and *bla*_{TEM}
280 concentrations revealed a significant correlation between the quantities of *bla*_{TEM} genes and
281 the counts of ampicillin-resistant *Enterobacteriaceae*. The observed scatter is probably due
282 partly to the inaccuracy of both techniques and to the fact that amplified *bla*_{TEM} genes may be
283 harbored by bacteria other than *Enterobacteriaceae* (13).

284 During our experiment to monitor *bla*_{TEM} genes excretion, we found that treated pigs
285 excreted more *bla*_{TEM} genes than control pigs. Moreover, as in the phenotypic evaluations, the
286 fecal excretion of *bla*_{TEM} genes showed large individual day-to-day fluctuations. As indicated
287 above, these fluctuations were correlated with counts of ampicillin-resistant
288 *Enterobacteriaceae*. Similarly, Belloc *et al.* (2) studied the effect of quinolone treatment on
289 selection and persistence of quinolone-resistant *E. coli* in swine fecal flora and observed great
290 variability both in the percentage of resistant strains and pattern of emergence of resistance. In

291 the present study, despite the great variability and the small number of pigs *per* mode of
292 treatment, at least two of the three modes of drug administration (i.e. intramuscular route and
293 oral route in fed pigs) could be differentiated by quantifying the *bla*_{TEM} genes excreted in
294 feces, but not by phenotypic evaluation. These results imply that a genotypic indicator can be
295 used advantageously as a complement to phenotypic approaches to quantitatively evaluate the
296 intestinal reservoir of resistance genes. For example, *bla*_{TEM} genes quantification has already
297 been used to evaluate ampicillin-induced selective pressure on the gut microbiota in dogs
298 (12).

299 Our results, showing that oral administration of ampicillin in fed pigs was associated
300 with the highest excretion level of fecal *bla*_{TEM} genes, are consistent with both our
301 pharmacokinetic measurements (not shown) and published data. These latter indicate that β -
302 lactam absorption following oral administration is largely incomplete in pigs (1, 14) and that
303 feeding decreases β -lactam absorption in pigs as in dogs (15) and humans (32). As a
304 consequence, these expected high concentrations of unabsorbed ampicillin in the intestine are
305 likely to exert great pressure on the gut microflora, and this all the more if ampicillin is
306 administered to fed pigs. Following intramuscular administration, ampicillin can gain access
307 to the gastrointestinal lumen by biliary excretion (9), which explains why the intramuscular
308 route was also associated with an increase in fecal *bla*_{TEM} genes excretion. Thus the
309 pharmacokinetic profiles of the three modes of ampicillin administration tested in the present
310 study were apparently different and resulted in different intestinal exposures.

311 In conclusion, our study indicates that fecal *bla*_{TEM} genes quantification might be a
312 useful tool to evaluate and discriminate the impact of different modes of ampicillin
313 administration on the gut microflora. In the future, this quantitative tool might help to
314 quantify the flux of resistance genes in epidemiological investigations.

315 **ACKNOWLEDGMENTS**

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

317 We thank Nathalie Arpaillange and Nadine Gauthier for their technical support and Jean-

318 Pierre Gau, Patrice Rouby, Joseph Maligoy and Jean Denjean for taking care of the animals.

319 **REFERENCES**

- 320 1. **Agerso, H., and C. Friis.** 1998. Bioavailability of amoxicillin in pigs. *J Vet*
321 *Pharmacol Ther* **21**:41-6.
- 322 2. **Belloc, C., D. N. Lam, J. L. Pellerin, F. Beaudeau, and A. Laval.** 2005. Effect of
323 quinolone treatment on selection and persistence of quinolone-resistant *Escherichia*
324 *coli* in swine faecal flora. *J Appl Microbiol* **99**:954-9.
- 325 3. **Bergeron, M. G., and M. Ouellette.** 1998. Preventing antibiotic resistance through
326 rapid genotypic identification of bacteria and of their antibiotic resistance genes in the
327 clinical microbiology laboratory. *J Clin Microbiol* **36**:2169-72.
- 328 4. **Blake, D. P., R. W. Humphry, K. P. Scott, K. Hillman, D. R. Fenlon, and J. C.**
329 **Low.** 2003. Influence of tetracycline exposure on tetracycline resistance and the
330 carriage of tetracycline resistance genes within commensal *Escherichia coli*
331 populations. *J Appl Microbiol* **94**:1087-97.
- 332 5. **Courvalin, P.** 1991. Genotypic approach to the study of bacterial resistance to
333 antibiotics. *Antimicrob Agents Chemother* **35**:1019-23.
- 334 6. **Davison, H. C., J. C. Low, and M. E. Woolhouse.** 2000. What is antibiotic
335 resistance and how can we measure it? *Trends Microbiol* **8**:554-9.
- 336 7. **Escoula, L., M. Coste, and G. Larrieu.** 1982. Small filter membrane bags for the
337 study of antibiotic action in the digestive tract: bioavailability and in situ efficacy of
338 ampicillin in the pig caecum. *Res Vet Sci* **33**:338-42.
- 339 8. **FEDESA.** 2000. Press release on the European Union conference: The microbial
340 threat. Copenhagen, FEDESA.
- 341 9. **Galtier, P., and J. Charpenteau.** 1979. Pharmacokinetics of ampicillin in pigs. *J. vet.*
342 *Pharmacol. Therap.* **2**:173-180.

- 343 10. **Gellin, G., B. E. Langlois, K. A. Dawson, and D. K. Aaron.** 1989. Antibiotic
344 resistance of gram-negative enteric bacteria from pigs in three herds with different
345 histories of antibiotic exposure. *Appl Environ Microbiol* **55**:2287-92.
- 346 11. **Hansen, W., and E. Yourassowsky.** 1984. Detection of beta-glucuronidase in
347 lactose-fermenting members of the family Enterobacteriaceae and its presence in
348 bacterial urine cultures. *J Clin Microbiol* **20**:1177-9.
- 349 12. **Harmoinen, J., S. Mentula, M. Heikkila, M. van der Rest, P. J. Rajala-Schultz, C.**
350 **J. Donskey, R. Frias, P. Koski, N. Wickstrand, H. Jousimies-Somer, E.**
351 **Westermarck, and K. Lindevall.** 2004. Orally administered targeted recombinant
352 Beta-lactamase prevents ampicillin-induced selective pressure on the gut microbiota: a
353 novel approach to reducing antimicrobial resistance. *Antimicrob Agents Chemother*
354 **48**:75-9.
- 355 13. **Heritage, J., N. Ransome, P. A. Chambers, and M. H. Wilcox.** 2001. A comparison
356 of culture and PCR to determine the prevalence of ampicillin-resistant bacteria in the
357 faecal flora of general practice patients. *J Antimicrob Chemother* **48**:287-9.
- 358 14. **Jensen, G. M., J. Lykkesfeldt, K. Frydendahl, K. Moller, and O. Svendsen.** 2004.
359 Pharmacokinetics of amoxicillin after oral administration in recently weaned piglets
360 with experimentally induced *Escherichia coli* subtype O149:F4 diarrhea. *Am J Vet*
361 *Res* **65**:992-5.
- 362 15. **Kung, K., B. R. Hauser, and M. Wanner.** 1995. Effect of the interval between
363 feeding and drug administration on oral ampicillin absorption in dogs. *J Small Anim*
364 *Pract* **36**:65-8.
- 365 16. **Livermore, D. M., F. Moosdeen, M. A. Lindridge, P. Kho, and J. D. Williams.**
366 1986. Behaviour of TEM-1 beta-lactamase as a resistance mechanism to ampicillin,
367 mezlocillin and azlocillin in *Escherichia coli*. *J Antimicrob Chemother* **17**:139-46.

- 368 17. **Maeda, H., C. Fujimoto, Y. Haruki, T. Maeda, S. Kokeyuchi, M. Petelin, H. Arai,**
369 **I. Tanimoto, F. Nishimura, and S. Takashiba.** 2003. Quantitative real-time PCR
370 using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*,
371 *Porphyromonas gingivalis*, *Prevotella intermedia*, tetQ gene and total bacteria. *FEMS*
372 *Immunol Med Microbiol* **39**:81-6.
- 373 18. **Mentula, S., T. Virtanen, A. Kanervo-Nordstrom, J. Harmoinen, E.**
374 **Westermarck, M. Rautio, P. Huovinen, and E. Kononen.** 2006. Relatedness of
375 *Escherichia coli* strains with different susceptibility patterns isolated from beagle dogs
376 during ampicillin treatment. *Int J Antimicrob Agents* **27**:46-50.
- 377 19. **NCCLS.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that
378 grow aerobically; Approved standard-Sixth Edition - Document M7-A6. NCCLS,
379 Wayne, PA.
- 380 20. **NCCLS.** 2003. Performance Standards for Antimicrobial Susceptibility Testing;
381 Thirteenth Informational Supplement- Document M100-S13 (M7) for use with
382 document M7-A6-MIC testing. NCCLS, Wayne, PA.
- 383 21. **Paterson, D. L.** 2006. Resistance in gram-negative bacteria: Enterobacteriaceae. *Am J*
384 *Infect Control* **34**:S20-8; discussion S64-73.
- 385 22. **Paterson, D. L., and R. A. Bonomo.** 2005. Extended-spectrum beta-lactamases: a
386 clinical update. *Clin Microbiol Rev* **18**:657-86.
- 387 23. **Phillips, I.** 1998. The 1997 Garrod Lecture. The subtleties of antibiotic resistance. *J*
388 *Antimicrob Chemother* **42**:5-12.
- 389 24. **Phillips, I., M. Casewell, T. Cox, B. De Groot, C. Friis, R. Jones, C. Nightingale,**
390 **R. Preston, and J. Waddell.** 2004. Does the use of antibiotics in food animals pose a
391 risk to human health? A critical review of published data. *J Antimicrob Chemother*
392 **53**:28-52.

- 393 25. **Poole, K.** 2004. Resistance to beta-lactam antibiotics. *Cell Mol Life Sci* **61**:2200-23.
- 394 26. **Salyers, A. A., A. Gupta, and Y. Wang.** 2004. Human intestinal bacteria as
395 reservoirs for antibiotic resistance genes. *Trends Microbiol* **12**:412-6.
- 396 27. **Smith, M. S., R. K. Yang, C. W. Knapp, Y. Niu, N. Peak, M. M. Hanfelt, J. C.**
397 **Galland, and D. W. Graham.** 2004. Quantification of tetracycline resistance genes in
398 feedlot lagoons by real-time PCR. *Appl Environ Microbiol* **70**:7372-7.
- 399 28. **Sorum, H., and M. Sunde.** 2001. Resistance to antibiotics in the normal flora of
400 animals. *Vet Res* **32**:227-41.
- 401 29. **Sullivan, A., C. Edlund, and C. E. Nord.** 2001. Effect of antimicrobial agents on the
402 ecological balance of human microflora. *Lancet Infect Dis* **1**:101-14.
- 403 30. **Sunde, M., K. Fossum, A. Solberg, and H. Sorum.** 1998. Antibiotic resistance in
404 *Escherichia coli* of the normal intestinal flora of swine. *Microb Drug Resist* **4**:289-99.
- 405 31. **Tenover, F. C.** 1989. DNA probes for antimicrobial susceptibility testing. *Clin Lab*
406 *Med* **9**:341-7.
- 407 32. **Welling, P. G.** 1989. Effects of food on drug absorption. *Pharmacol Ther* **43**:425-41.
- 408 33. **Wiuff, C., J. Lykkesfeldt, O. Svendsen, and F. M. Aarestrup.** 2003. The effects of
409 oral and intramuscular administration and dose escalation of enrofloxacin on the
410 selection of quinolone resistance among *Salmonella* and coliforms in pigs. *Res Vet Sci*
411 **75**:185-93.
- 412 34. **Yan, S. S., and J. M. Gilbert.** 2004. Antimicrobial drug delivery in food animals and
413 microbial food safety concerns: an overview of in vitro and in vivo factors potentially
414 affecting the animal gut microflora. *Adv Drug Deliv Rev* **56**:1497-521.
- 415 35. **Yu, Z., F. C. Michel, Jr., G. Hansen, T. Wittum, and M. Morrison.** 2005.
416 Development and application of real-time PCR assays for quantification of genes
417 encoding tetracycline resistance. *Appl Environ Microbiol* **71**:6926-33.

418 **FIGURE LEGENDS**

419

420 FIG. 1. Standard curves calculated with the control plasmid diluted in Tris -EDTA buffer (●)
421 or in DNA extracted from swine feces (◆). Amplification was repeated four times for each
422 dilution.

423

424 FIG. 2. a) Percentage of ampicillin resistant *Enterobacteriaceae* for each mode of ampicillin
425 administration. These percentages were calculated from the total counts of
426 *Enterobacteriaceae* in the absence or presence of ampicillin (16 µg/mL). b) Percentage of
427 ampicillin-resistant *E. coli* (i.e. with MIC above 16 µg/mL), for each mode of ampicillin
428 administration. Ampicillin susceptibility was tested at each sampling point on 20 isolates
429 from each pig. Treated pigs had received ampicillin at 20 mg/kg from day 0 to day 6 by
430 intramuscular route (▲) (n=4), oral route in fasted (■) (n=4) or fed (□) (n=4) pigs. 6 pigs
431 were used as a control (●). Values are means and error bars represent standard deviations.

432

433 FIG. 3. Copy number of *bla*_{TEM} genes per g of feces detected by real-time PCR for each pig.
434 Ampicillin was administered at 20 mg/kg from day 0 to day 6. Modes of administration were:
435 a) oral route in fed pigs (n=4), b) oral route in fasted pigs (n=4), c) intramuscular route (n=4).
436 d) 6 pigs were used as a control.

437

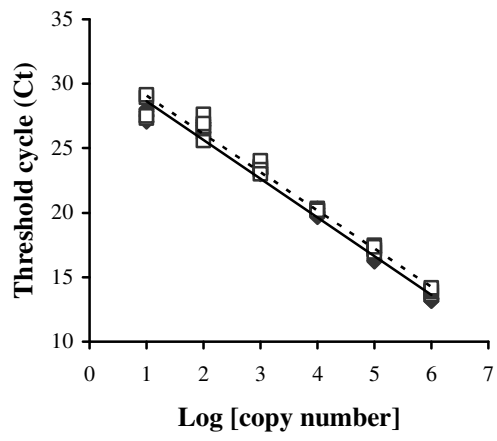
438 FIG. 4. Copy number of *bla*_{TEM} genes per g of feces for each mode of ampicillin
439 administration. Treated pigs had received ampicillin at 20 mg/kg from day 0 to day 6 by
440 intramuscular route (▲) (n=4), oral route in fasted (■) (n=4) or fed (□) (n=4) pigs. 6 pigs
441 were used as a control (●). Values are means and error bars represent standard deviations.

442

443 FIG. 5. Relationship between the log of the *bla*_{TEM} copy number/g feces and the log of counts
444 of ampicillin-resistant *Enterobacteriaceae* /g of feces.

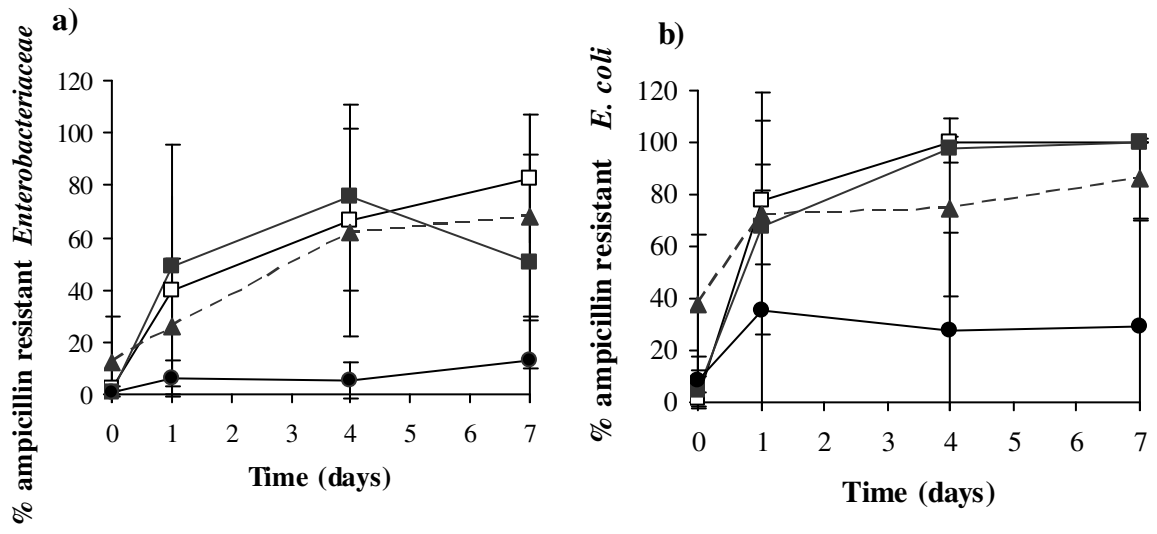
445

446 FIGURE 1
447



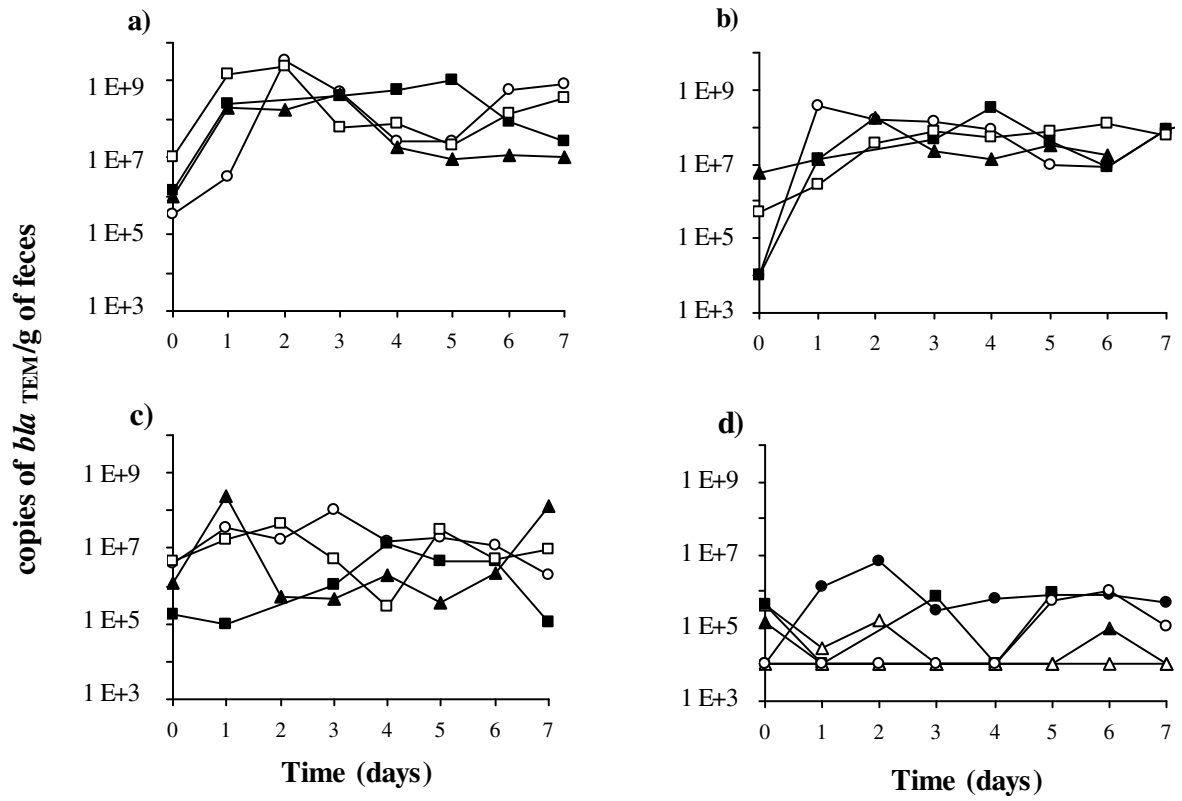
448
449
450

451 FIGURE 2
452



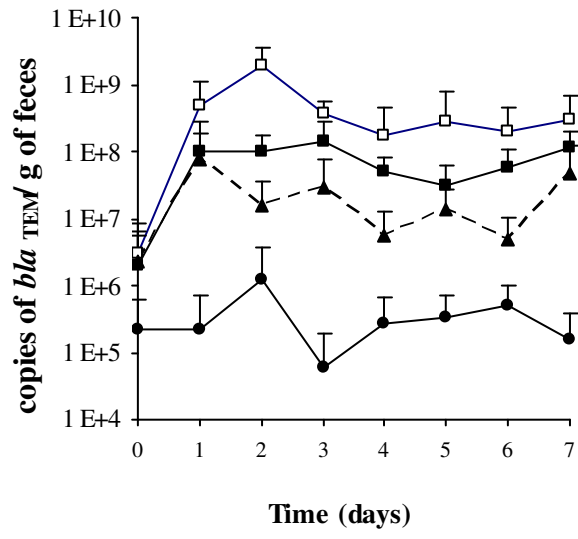
453
454
455
456

457 FIGURE 3
458



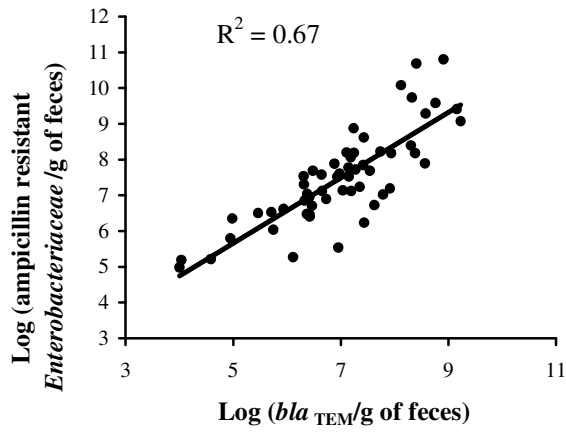
459
460

461 FIGURE 4
462



463
464
465

466 FIGURE 5
467



468
469

470