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

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ABSTRACT

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

INTRODUCTION

The major mechanism of resistance to β-lactam antibiotics in Gram-negative bacteria results from the production of β-lactamases. Most of these are coded by the plasmid-mediated $bla_{\text{TEM-1}}$ gene (19, 28). The continuous introduction of new β-lactam antibiotics with different activity spectra in human medicine has led to the selection of β-lactamase mutations, which confer resistance to the newly-developed β-lactam antibiotics (25). β-lactam antibiotics are also used in veterinary medicine where they contribute to the selective pressure that leads to the emergence and diffusion of intestinal bacteria harboring resistance genes. Thus, commensal bacteria in the gut form a reservoir of antibiotic resistance genes potentially transmissible to humans *via* the food-chain and the environment (27, 29, 34).

Antimicrobial resistance in food animals deserves special attention. One of the most heavily medicated sectors is pig-farming, world-wide antibiotic consumption in pigs accounting for 60% of the antibiotics used in animals (10). A relationship has been demonstrated between the high use of antimicrobials in pig herds and increased occurrence of resistant bacterial strains in their digestive tracts (4, 13, 34, 37).When antibiotics are administered to pigs, both the level and time-development of antibiotic exposure of the intestinal microflora are dependent on the mode of drug administration (38). This exposure is a key determinant of antibiotic resistance development in the gut flora, and the relation between antibiotic dosage regimen and resistance merits attention. The impact of different antibiotic dosage regimens on the emergence of resistance must be evaluated by appropriate quantitative indicators of the resistance level. Traditionally, this has involved phenotypic methods that measure bacterial antibiotic susceptibility (32). In addition, quantitative PCR has 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 (15, 20, 31, 39).

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

MATERIALS AND METHODS

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

For phenotypic evaluation of ampicillin resistance, fecal samples were taken from each pig, by digital manipulation or immediately after spontaneous defecation, at days 0 (before ampicillin administration), 1, 4, and 7. The samples were immediately transferred to the laboratory and the *Enterobacteriaceae* were counted. For the quantification of bla_{TEM} genes in feces by real-time PCR, feces of each pig were collected two or three times before the treatment. The value given for day 0 is the mean of these samplings. Feces were then 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. MacConkey agar is classically used for selective
106 growth of *Enterobacteriaceae* (7, 8, 11, 30). *Enterobacteriaceae* growing in the presence of
107 16 µg/mL of ampicillin were classified as resistant. This concentration corresponds to the
108 MIC breakpoint value (MIC ≥ 32 µg/mL) proposed by the CLSI (23) and the French Society
109 of Microbiology (http://www.sfm.asso.fr). The plates were incubated at 37°C for 24 h.
110 *Enterobacteriaceae* counts from both plates were used to calculate the percentage of resistant
111 *Enterobacteriaceae* at each sampling time.

112 For each sample, 20 colonies were randomly picked on the MacConkey plates without
113 ampicillin and stored at -80°C until assayed. These colonies were considered as *E. coli* on the
114 basis of β-glucuronidase production using TBX agar (Tryptone Bile X-glucuronide agar, AES
115 laboratoire, Bruz, France) (14). Only a few colonies were β-glucuronidase negative. All β-
116 glucuronidase negative isolates and a portion of β-glucuronidase positive isolates were tested
117 by the API 20E *Enterobacteriaceae* identification system (bioMérieux, Marcy l'Etoile,
118 France) to confirm their identification. For MICs determination, ampicillin susceptibility was
119 tested by microdilution broth dilution method according to the recommendations reported by
120 the CLSI (22). The control strain was *E. coli* ATCC 25922.

121 **Bacteria and growth conditions.** *E. coli* JS238[pOFX326], the plasmid of which
122 carries a monocopy of the target gene $bla_{\text{TEM-1}}$, was used to optimize real-time PCR, assess

sensitivity and generate quantification standards. The strain was cultured in Mueller-Hinton broth containing ampicillin at the concentration of 50 µg/mL at 37°C overnight.

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

Design of primers. The PCR primers were designed with Pimer 3 and Oligo Analyser. The specificity of the sequence was further checked against all the available GenBank DNA sequences. The forward and reverse primers chosen for bla_{TEM} genes quantification were 5'-TTCCTGTTTTTGCTCACCCAG-3' and 5'-CTCAAGGATCTTACCGCTGTTG-3', respectively. These primers amplify a 112 bp segment of the $bla_{\text{TEM-1D}}$ gene (GeneBank accession number AF 1888200) from nucleotide positions 270 to 382. A 100% homology was demonstrated with 130 bla_{TEM} genes for which the nucleotide sequence was available, except for TEM-60.

Real-time PCR assay. The PCR amplification was performed in a 25 µL reaction mixture with a SYBR Green PCR Core Reagents kit (Perkin Elmer Biosystems, Foster City, USA). The reaction mixture contained 5 µL of test DNA solution, 2.5 µL of 10X SYBR Green PCR Buffer, 1.6 µL of a deoxynucleoside triphosphate solution (2.5 mM each of dATP, dCTP and dGTP and 5 mM of dUTP), 0.25 µL of each primer (20 µM), 4 µL of 25 mM $MgCl_2$, 11.275 µL of Ultra Pure Water (Qbiogene, Montréal, Canada) and 0.125 µL of AmpliTaq Gold® DNA Polymerase, LD (5 U/µL) (Perkin Elmer Biosystems). Amplification was performed using a GeneAmp® PCR System 5700 thermocycler (Perkin Elmer Biosystems) with the following conditions: 95°C for 10 min followed by 45 cycles of 15

seconds at 95°C and 1 minute at 60°C. A standard curve with three replicates of the control plasmid pOFX326 diluted in Tris-EDTA buffer was generated for each PCR assay. All sample PCRs were done in duplicate. The samples were checked for absence of background levels of PCR-inhibiting compounds by spiking DNA extracted from the samples with target DNA and subjecting these spiked DNA samples to real-time PCR both undiluted and diluted (1:10).

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

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

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

where Y_{ijk} is the measure of resistance for pig k undergoing ampicillin administration with modality i at day j, μ the overall mean, M_i the differential effect of treatment i, D_j the differential effect of day j, $M{*}D_{ij}$ the corresponding interaction, $A_k\big|_{M_i}$ the differential effect of animal k nested within treatment i and ε_{ijk} an error term. Y, the measure of resistance, was monitored in various ways. For the phenotypic evaluation of resistance, Y was the log-transformed percentage of the resistant *Enterobacteriaceae* population or the log-transformed

173 percentage of *E. coli* isolates with MIC > 16 μg/ml. For the genotypic evaluation, Y was the
174 log-transformed quantity of bla_{TEM} genes. Multiple comparisons were performed using the
175 Tukey test. The selected level of significance was $P<0.05$.

RESULTS

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

201 **Phenotypic evaluation of ampicillin resistance.** Average percentages of ampicillin-
202 resistant *Enterobacteriaceae* for each treatment group are given in Fig. 2a. The average
203 percentage of resistant *Enterobacteriaceae* ranged from 0.9% to 12% before ampicillin
204 administration. On the first day of treatment, it rose to 26% for the intramuscular route and to
205 40% and 49% for the oral routes in fed and fasted pigs respectively. By days 4 and 7, the level
206 of resistance exceeded 50% in all treated groups. In contrast, the level of resistance in the
207 control group remained below 13% at all times. Treated animals excreted significantly higher
208 percentages of resistant *Enterobacteriaceae* compared to the control group ($P<0.05$).
209 However, no significant differences were observed between the three modes of drug
210 administration ($P>0.05$). Furthermore, Fig. 2a shows the high inter-individual variability
211 within each group.
212 Ampicillin resistance was also monitored from the percentage of resistant *E. coli*
213 isolates for each treatment group (Fig. 2b). The average percentage of resistant *E. coli* ranged
214 from 1% to 38% before ampicillin administration. At day 1 of treatment, about 70% of
215 isolates were resistant, whatever the mode of drug administration. By days 4 and 7, nearly all
216 the isolates, whatever the dosage regimen, were resistant. In contrast, the percentages of
217 resistant *E. coli* remained below 36% in the control group. Statistical analysis indicated that
218 oral administration in fed pigs led to a higher fecal excretion of resistant *E. coli* than in
219 control pigs ($P<0.05$). The two other dosage regimens did not differ significantly from the
220 control group due to the great heterogeneity of the control group data ($P>0.05$). High inter-
221 individual variability also existed within each ampicillin-treated group.
222 **Genotypic evaluation of ampicillin resistance.** Ampicillin resistance in feces was
223 measured by bla_{TEM} genes quantification using the validated PCR assay. bla_{TEM} genes copy
224 numbers per gram of wet feces were measured on each day of treatment for each pig (Fig. 3).
225 The baseline values for all pigs were below 10^7 copies/g of feces. bla_{TEM} quantities increased

after ampicillin administration. The between-day fluctuations for a given animal were large. The bla_{TEM} quantities for the oral routes fluctuated between 10^7 and 10^9 copies/g of feces, but only between 10^5 and 10^8 copies/g of feces for the intramuscular route. Two fed pigs treated by oral route excreted the highest bla_{TEM} quantities with values above 10^9 copies/g of feces. The bla_{TEM} quantities for the control group were lower than those of the three ampicillin-treated groups and fluctuated between 10^4 and 10^6 copies/g of feces.

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

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

DISCUSSION

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

Antibiotic impact on the gut microflora is generally measured by phenotypic evaluation of antibiotic resistance on a limited bacterial population, either using isolates of indicator bacteria or families of bacteria. *E. coli* and *Enterobacteriaceae* are good candidates for studies of the antibiotic resistance level of the fecal flora and are commonly used for this in pigs (32). These bacteria are easily culturable and their isolation is facilitated by specific culture media. In the present experiment, results obtained with the two phenotypic indicators of ampicillin resistance implied that all treatments had a similar negative impact on the gut microflora with the emergence of a high level of resistance at all three dosage regimens. These results are consistent with those of previous studies demonstrating that ampicillin treatment could have a marked effect on the level of resistance in intestinal microbiota of several species (9, 21, 33). Nevertheless, the phenotypic indicators commonly used to assess antibiotic resistance exhibit methodological features that impact both their metrological performances and relevance. Firstly, the selected indicator bacteria must be cultured and the reliability of results has been questioned due to considerable variation originating from the

268 culture medium, bacterial inoculum, antibiotic preparation and incubation conditions (26).
269 Secondly, the isolates might not be representative of the whole population of bacteria (6).
270 These limits impair the sensitivity and precision of phenotypic indicators for the assessment
271 of resistance levels and have prompted investigators to develop molecular techniques as
272 alternatives, in particular quantitative PCR (15, 20, 31, 39).

273 Molecular techniques can be used to reveal the presence of genetic determinants without
274 bacterial cultivation and irrespective of the bacterial species carrying these genetic
275 determinants (5, 35). However, a requisite to this approach is the knowledge of the underlying
276 resistance mechanisms, and when few genes are involved in resistance, they may provide
277 candidates for resistance markers (3). *bla*$_{TEM}$ genes code for the most commonly encountered
278 β-lactamases in Gram-negative bacteria (24). We therefore developed and validated a real-
279 time PCR assay to quantify *bla*$_{TEM}$ genes in swine feces. This PCR assay was suitable for
280 quantification of *bla*$_{TEM}$ genes from 10^4 to 10^9 copies/g of feces.

281 Examination of the agreement between resistant *Enterobacteriaceae* counts and *bla*$_{TEM}$
282 concentrations revealed a significant correlation between the quantities of *bla*$_{TEM}$ genes and
283 the counts of ampicillin-resistant *Enterobacteriaceae*. The observed scatter is probably due
284 partly to the inaccuracy of both techniques and to the fact that amplified *bla*$_{TEM}$ genes may be
285 harbored by bacteria other than *Enterobacteriaceae* (16).

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

293 the present study, despite the great variability and the small number of pigs *per* mode of
294 treatment, at least two of the three modes of drug administration (i.e. intramuscular route and
295 oral route in fed pigs) could be differentiated by quantifying the bla_{TEM} genes excreted in
296 feces, but not by phenotypic evaluation. These results imply that a genotypic indicator can be
297 used advantageously as a complement to phenotypic approaches to quantitatively evaluate the
298 intestinal reservoir of resistance genes. For example, bla_{TEM} genes quantification has already
299 been used to evaluate ampicillin-induced selective pressure on the gut microbiota in dogs
300 (15).

301 Our results, showing that oral administration of ampicillin in fed pigs was associated
302 with the highest excretion level of fecal bla_{TEM} genes, are consistent with both our
303 pharmacokinetic measurements (not shown) and published data. These latter indicate that β-
304 lactam absorption following oral administration is largely incomplete in pigs (1, 17) and that
305 feeding decreases β-lactam absorption in pigs as in dogs (18) and humans (36). As a
306 consequence, these expected high concentrations of unabsorbed ampicillin in the intestine are
307 likely to exert great pressure on the gut microflora, and this all the more if ampicillin is
308 administered to fed pigs. Following intramuscular administration, ampicillin can gain access
309 to the gastrointestinal lumen by biliary excretion (12), which explains why the intramuscular
310 route was also associated with an increase in fecal bla_{TEM} genes excretion. Thus the
311 pharmacokinetic profiles of the three modes of ampicillin administration tested in the present
312 study were apparently different and resulted in different intestinal exposures.

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

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FIGURE LEGENDS

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

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

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

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

458 FIG. 5. Relationship between the log of the *bla*$_{TEM}$ copy number/g feces and the log of counts

459 of ampicillin-resistant *Enterobacteriaceae* /g of feces.

460

461 FIGURE 1
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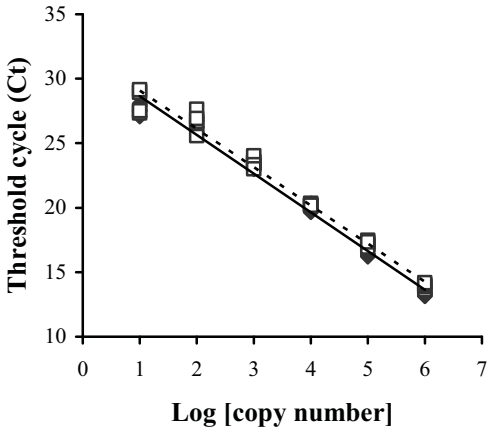

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466 FIGURE 2
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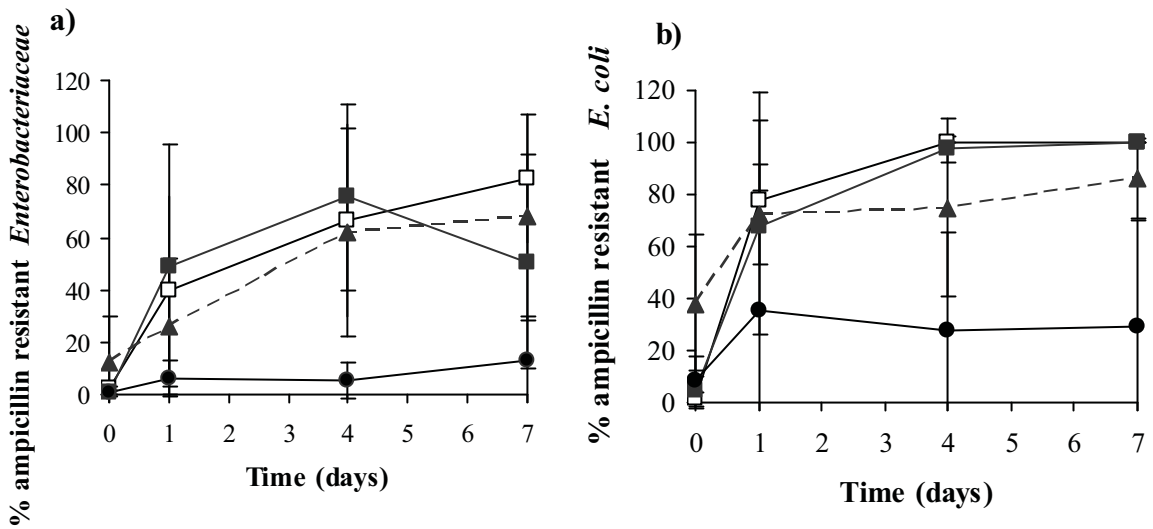

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472 FIGURE 3
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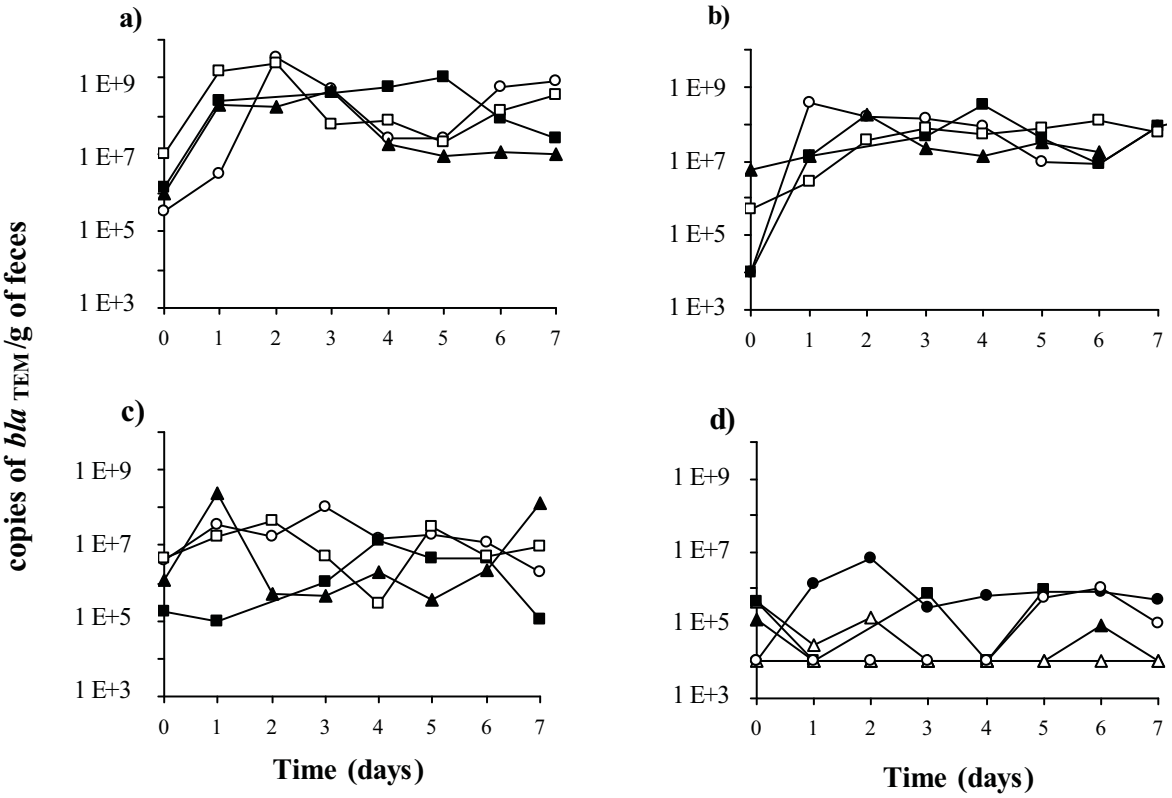

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475

476 FIGURE 4
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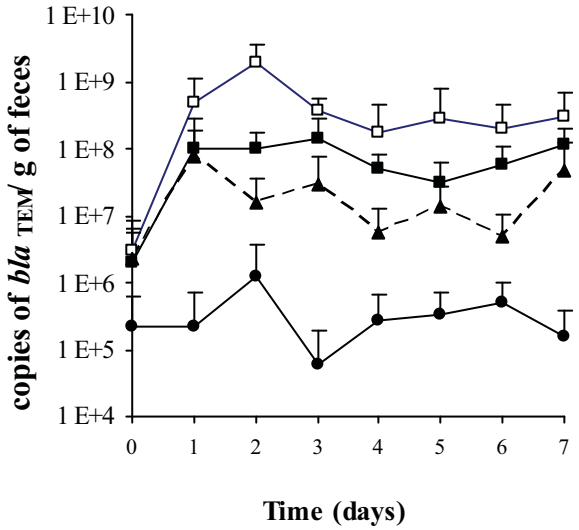

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481 FIGURE 5
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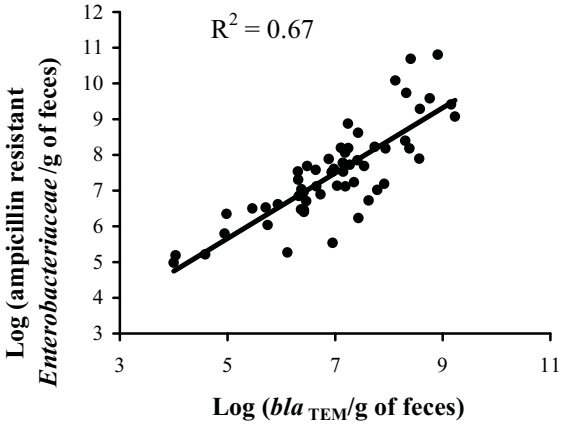

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