APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2007, p. 4785–4790 Vol. 73, No. 15
0099-2240/07/$08.00+0 doi:10.1128/AEM.00252-07

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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Received 31 January 2007/Accepted 3 June 2007

The aim of this study was to assess the impact of three ampicillin dosage regimens on ampicillin resistance among *Enterobacteriaceae* recovered from swine feces by use of phenotypic and genotypic approaches. Phenotypically, ampicillin resistance was determined from the percentage of resistant *Enterobacteriaceae* and MICs of *Escherichia 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 orally to fed or fasted pigs for 7 days at 20 mg/kg of body weight. The average percentage of resistant *Enterobacteriaceae* before treatment was between 2.5% and 12%, and bla_{TEM} gene quantities were below 10^7 copies/g of feces. By days 4 and 7, the percentage of resistant *Enterobacteriaceae* exceeded 50% in all treated groups, with some highly resistant strains (MIC of >256 μg/ml). In the control group, bla_{TEM} gene quantities fluctuated between 10^4 and 10^6 copies/g of feces, whereas they fluctuated between 10^6 to 10^8 and 10^7 to 10^9 copies/g of feces for the intramuscular and oral routes, respectively. Whereas phenotypic evaluations did not discriminate among the three ampicillin dosage regimens, bla_{TEM} gene quantification was able to differentiate between the effects of two routes of ampicillin administration. Our results suggest that fecal bla_{TEM} gene 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.

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_{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, with worldwide 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 the 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 gene surveillance because (i) it is sensitive, (ii) unambiguous standard curves can be used to quantify the resistance genes from various matrices, and (iii) no bacterial cultivation is required (15, 20, 31, 39).

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

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▽ Published ahead of print on 8 June 2007.

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 of body weight for 7 days (from day 0 to day 6) following three modalities: the intramuscular route, the oral route in fasted pigs, or the oral route in fed pigs. The design schedule consisted of three successive series of six animals receiving ampicillin treatments as follows: intramuscular ($n = 2$), oral route under fed conditions ($n = 2$), and control without treatment ($n = 2$) in the first series; intramuscular ($n = 2$), oral route under fasted conditions ($n = 2$), and control without treatment ($n = 2$) in the second series; oral route under fed conditions ($n = 2$), oral route under fasted conditions ($n = 2$), and control without treatment ($n = 2$) in the third series. Six pigs were used in the control group and four 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 h before ampicillin administration and fed 4 h after ampicillin administration. Ampicillin was administered to fed pigs 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 hundred milligrams of feces from each sample was frozen in liquid nitrogen and stored at −80°C until assayed.

Phenotypic evaluation of ampicillin resistance. Feces (5 g) from each pig were homogenized with 45 ml of peptone water, including 30% glycerol, with a Bag-Mixer (Interscience, St. Nom, France). Tenfold serial dilutions of the filtrate were prepared, and 100-μl samples of the dilutions were spread on MacConkey plates (AEB 151602; AES, Ker Lann, France) containing 0 and 16 μg/ml of ampicillin. MacConkey agar is classically used for selective growth of *Enterobacteriaceae* (7, 8, 11, 30). *Enterobacteriaceae* growing in the presence of 16 μg/ml of ampicillin were classified as resistant. This concentration corresponds to the MIC breakpoint value (MIC of ≥32 μg/ml) proposed by the CLSI (23) and the French Society for Microbiology (http://www.sfm.asso.fr). The plates were incubated at 37°C for 24 h. *Enterobacteriaceae* counts from both plates were used to calculate the percentage of resistant *Enterobacteriaceae* at each sampling time.

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

Bacteria and growth conditions. *E. coli* JS238(pOFX326), the plasmid of which carries a monocopy of the target gene $bla_{\mathrm{TEM\text{-}1}}$, 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 kit (QIAGEN, Hilden, Germany). Quality was assessed by migration on gel electrophoresis in 1% agarose after digestion with HindIII, and concentration was assessed by spectrophotometry at 260 nm. The QIAamp DNA stool kit (QIAGEN, Hilden, Germany) was used to extract DNA from feces according to the manufacturer's recommendations. For each series of extractions, a positive control and a negative control were coextracted and subjected to real-time PCR.

Design of primers. The PCR primers were designed with Primer 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} gene quantification were 5′-TTCCTGTTTTTGCTCACCCAG-3′ and 5′-CTCAAGGATCTTACCGCTGTTG-3′, respectively. These primers amplify a 112-bp segment of the $bla_{\mathrm{TEM\text{-}1D}}$ gene (GenBank 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 reagent kit (PerkinElmer Biosystems, Foster City, CA). The reaction mixture contained 5 μl of test DNA solution, 2.5 μl of 10× 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 ultrapure water (Qbiogene, Montréal, Canada), and 0.125 μl of AmpliTaq Gold DNA polymerase, LD (5 U/μl) (PerkinElmer Biosystems). Amplification was performed using a GeneAmp PCR system 5700 thermocycler (PerkinElmer Biosystems) with the following conditions: 95°C for 10 min followed by 45 cycles of 15 seconds at 95°C and 1 min 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 the 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) were 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 and 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). Changes in the level of ampicillin resistance were analyzed using a generalized linear mixed-effects model with the following equation:

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

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, $\mathrm{A_k}|_{\mathrm{M_i}}$ 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 percentage of *E. coli* isolates with MICs of >16 μg/ml. For the genotypic evaluation, Y was the log-transformed quantity of bla_{TEM} genes. Multiple comparisons were performed using the Tukey test. The selected level of significance was a P value of <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 melting temperature of 81°C (data not shown). Despite the use of highly purified AmpliTaq Gold DNA polymerase, analysis of the ultrapure water melting curves revealed contamination and thus restricted the PCR quantification limit (data not shown). Figure 1 shows the two standard curves: the relation between C_T (cycle threshold) values and the logarithm of bla_{TEM} concentration was linear from 10 to 10^6 copies/μl. The determination coefficients (r^2) were 0.996 in Tris-EDTA and 0.985 in swine fecal DNA. The closeness between these standard curves indicated that the complex fecal DNA environ-

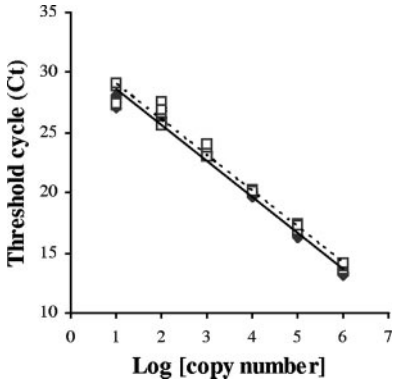

FIG. 1. Standard curves calculated with the control plasmid diluted in Tris-EDTA buffer (♦) or in DNA extracted from swine feces (□). Amplification was repeated four times for each dilution.

ment did not affect amplification sensitivity or performance. The intra- and interday 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 to 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/gram of feces and dilution factors of the JS238(pOFX326) solution was high ($r^2 = 0.904$). 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.

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

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

Genotypic evaluation of ampicillin resistance. Ampicillin resistance in feces was measured by bla_{TEM} gene quantification using a validated PCR assay. bla_{TEM} gene copy numbers per gram of wet feces were measured on each day of treatment for each pig (Fig. 3). 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

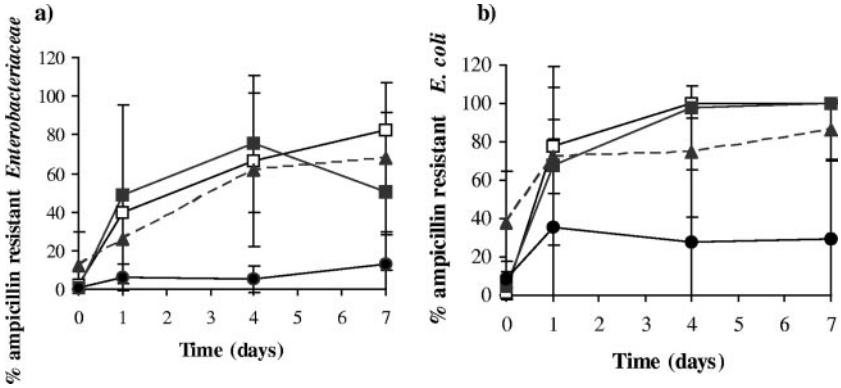

FIG. 2. (a) Percentages of ampicillin-resistant *Enterobacteriaceae* for each mode of ampicillin administration. These percentages were calculated from the total counts of *Enterobacteriaceae* in the absence or presence of ampicillin (16 μg/ml). (b) Percentages of ampicillin-resistant *E. coli* isolates (i.e., with MICs 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 received ampicillin at 20 mg/kg from day 0 to day 6 by the intramuscular route (▲) ($n = 4$), by the oral route in fasted pigs (■) ($n = 4$), or by the oral route in fed pigs (□) ($n = 4$). Six pigs were used as a control (●). Values are the means of the results, and error bars represent the standard deviations.

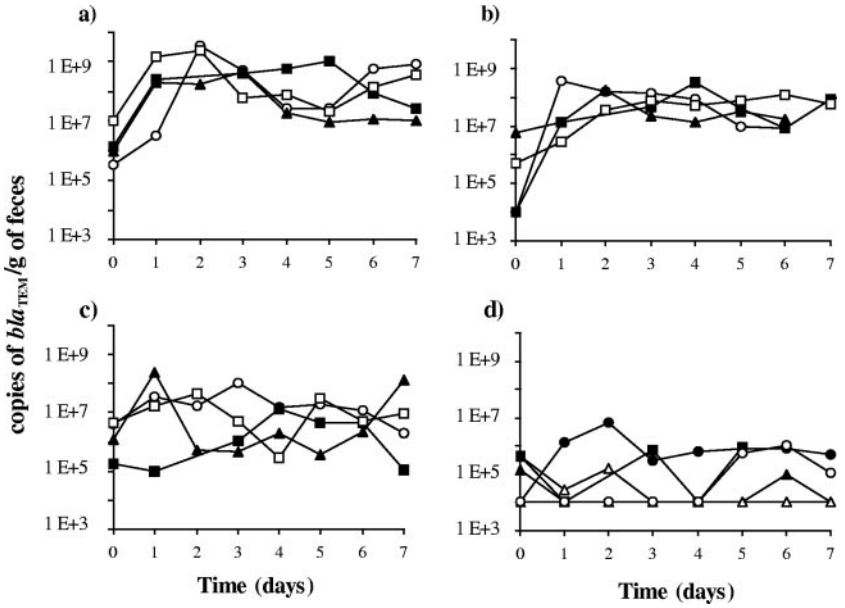

FIG. 3. Copy numbers of bla_{TEM} genes per gram of feces detected by real-time PCR for each pig. Ampicillin was administered at 20 mg/kg from day 0 to day 6. Modes of administration were the oral route in fed pigs ($n = 4$) (a), the oral route in fasted pigs ($n = 4$) (b), and the intramuscular route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

of the three ampicillin-treated groups and fluctuated between 10^4 and 10^6 copies/g of feces.

Figure 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 that in 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. Figure 5 shows a significant correlation ($r^2 = 0.67$) between the quantities of bla_{TEM} genes and the counts of ampicillin-resistant *Enterobacteriaceae*.

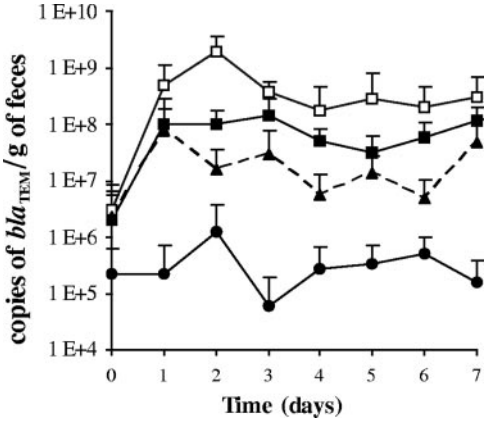

FIG. 4. Copy numbers of bla_{TEM} genes per gram of feces for each mode of ampicillin administration. Treated pigs received ampicillin at 20 mg/kg from day 0 to day 6 by the intramuscular route (▲) ($n = 4$), by the oral route in fasted pigs (■) ($n = 4$), or by the oral route in fed pigs (□) ($n = 4$).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

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 the 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 (on day 1 of treatment) to a large increase in the level of ampicillin resistance in the fecal microflora. In addition, the results suggested that the quanti-

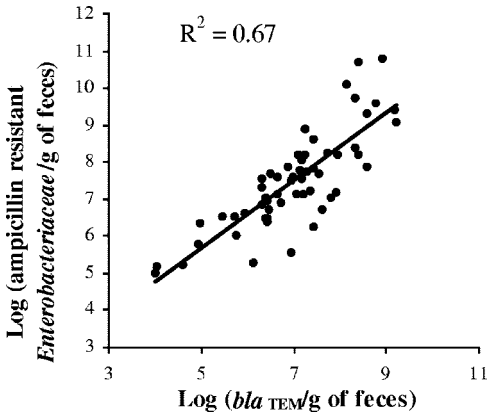

FIG. 5. Relationship between the log of the bla_{TEM} copy number/gram of feces and the log of counts of ampicillin-resistant *Enterobacteriaceae*/gram of feces.

tative 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 by 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 purpose 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 similar negative impacts on the gut microflora, with the emergence of a high level of resistance with 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 the 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 their relevance. First, the selected indicator bacteria must be cultured, and the reliability of results has been questioned due to considerable variation originating from the culture medium, bacterial inoculum, antibiotic preparation, and incubation conditions (26). Second, the isolates might not be representative of the whole population of bacteria (6). These limits impair the sensitivity and precision of phenotypic indicators for the assessment of resistance levels and have prompted investigators to develop molecular techniques as alternatives—in particular, quantitative PCR (15, 20, 31, 39).

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

Examination of the agreement between resistant *Enterobacteriaceae* counts and bla_{TEM} concentrations revealed a significant correlation between the quantities of bla_{TEM} genes and the counts of ampicillin-resistant *Enterobacteriaceae*. The observed scatter is probably due in part to the inaccuracy of both techniques and to the fact that amplified bla_{TEM} genes may be harbored by bacteria other than *Enterobacteriaceae* (16).

During our experiment to monitor bla_{TEM} gene excretion, we found that treated pigs excreted more bla_{TEM} genes than control pigs. Moreover, as in the phenotypic evaluations, the fecal excretion of bla_{TEM} genes showed large, individual, day-to-day fluctuations. As indicated above, these fluctuations were correlated with counts of ampicillin-resistant *Enterobacteriaceae*. Similarly, Belloc et al. (2) studied the effect of quinolone treatment on the selection and persistence of quinolone-resistant *E. coli* in swine fecal flora and observed great variability in both the percentages of resistant strains and the patterns of emergence of resistance. In the present study, despite the great variability and the small number of pigs per mode of treatment, at least two of the three modes of drug administration (i.e., the intramuscular route and the oral route in fed pigs) could be differentiated by quantifying the bla_{TEM} genes excreted in feces, but not by phenotypic evaluation. These results imply that a genotypic indicator can be used advantageously as a complement to phenotypic approaches to quantitatively evaluate the intestinal reservoir of resistance genes. For example, bla_{TEM} gene quantification has already been used to evaluate ampicillin-induced selective pressure on the gut microbiota in dogs (15).

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

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

ACKNOWLEDGMENTS

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

We thank Nathalie Arpaillange and Nadine Gauthier for their technical support and Jean-Pierre Gau, Patrice Rouby, Joseph Maligoy, and Jean Denjean for taking care of the animals.

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