Dynamics and Diversity of *Escherichia coli* in Animals and System Management of the Manure on a Commercial Farrow-to-Finish Pig Farm

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The objective of this study was to determine the dynamics and diversity of *Escherichia coli* populations in animal and environmental lines of a commercial farrow-to-finish pig farm in Spain along a full production cycle (July 2008 to July 2009), with special attention to antimicrobial resistance and the presence of integrons. In the animal line, a total of 256 isolates were collected from pregnant sows (10 samples and 20 isolates), 1-week-old piglets (20 samples and 40 isolates), unweaned piglets (20 samples and 38 isolates), growers (20 samples and 40 isolates), and the finishers’ floor pen (6 samples and 118 isolates); from the underfloor pits and farm slurry tank environmental lines, 100 and 119 isolates, respectively, were collected. Our results showed that *E. coli* populations in the pig fecal microbiota and in the farm environment are highly dynamic and show high levels of diversity. These issues have been proven through DNA-based typing data (repetitive extragenic palindromic PCR [REP-PCR]) and phenotypic typing data (antimicrobial resistance profile comprising 19 antimicrobials). Clustering of the sampling groups based on their REP-PCR typing results showed that the spatial features (the line) had a stronger weight than the temporal features (sampling week) for the clustering of *E. coli* populations; this weight was less significant when clustering was performed based on resistotypes. Among animals, finishers harbored an *E. coli* population different from those of the remaining animal populations studied, considering REP-PCR fingerprints and resistotypes. This population, the most important from a public health perspective, demonstrated the lowest levels of antimicrobial resistance and integron presence.

*Escherichia coli* is widely distributed among different ecological niches as a commensal or pathogenic bacterium. *E. coli* is the predominant facultative aerobic bacterium in the gastrointestinal tract of humans and many mammals (1, 2), and it is frequently used to represent commensal Gram-negative bacteria when studying antimicrobial resistance in farm animals (3, 4).

Some classical papers devoted to the ecology of intestinal *E. coli* in healthy pigs (5–9) have highlighted their continuous changes, indicating that individual animals, gut localization, and age, among other factors, determine the diversity of *E. coli*. Mainly serotyping (5–7) but also biotyping (8) and analyses of virulence traits (9) have been used as typing methodologies. More recently, manure (10, 11) and antimicrobial resistance (12–14) have been included in studies of the dynamics of *E. coli* in commercial farms, showing that antimicrobial resistance patterns in pig farms differ depending on the age of the animal and the farm type (15, 16). Other typing methodologies were also applied; among them, repetitive extragenic palindromic PCR (REP-PCR) was frequently used as a typing method (10, 11).

Integrons are genetic platforms involved in the spread of different previously captured gene cassettes that encode antimicrobial resistance determinants (17–19). Integrons have no specific mobilization machinery, although they are usually associated with other mobile genetic platforms, like transposons and/or plasmids, allowing them to be defined as mobile integrons; although classes 1 and 2 are the most frequently detected in many bacterial species, and especially in *E. coli*, all classes are associated with antimicrobial resistance determinants (19).

From a public health perspective, intestinal *E. coli* isolates of healthy finishers arriving at the slaughterhouse pose a major concern given that they could advance in the food chain through pork meat and derivatives. Furthermore, manure isolates can potentially be spread on land and in water, reaching humans indirectly. In both cases, antimicrobial-resistant *E. coli* isolates are of special concern because of their putative role as a source of antimicrobial resistance determinants that could be transferred to human-pathogenic bacteria.

The general objective of this study was to expand our knowledge of the population dynamics and diversity of *E. coli* in animals and facilities of farrow-to-finish pig farms throughout a full production cycle, paying particular attention to antimicrobial resistance and the presence of integrons.

MATERIALS AND METHODS

Description of the farm. The study was conducted from July 2008 to July 2009 in a commercial farrow-to-finish pig farm located in the Spanish Autonomous Community of Castilla-León. The farm had about 375 breeding sows and produced about 6,200 finishers per year plus about 2,000 growers per year for other finishing farms. The farm had five buildings (one for pregnancy and maternity, one for growers, one for growers and finishers, one for finishers, and one for repositioning). All pens had slotted floors. Animal slurries were collected in underfloor pits (UP) and then directed to an outside farm storage tank (FST). The tank was made of concrete and was partially emptied every 1 to 2 weeks.
Samples and sampling design. Fecal samples from individual animals and farm facilities (floor of finishers’ pens, UP of rooms, and FST) were taken during a meat production cycle. At week zero, 100 g of feces from the rectum was collected from each of 10 sows in their last week of pregnancy, which were housed in two adjacent rooms (7 in room A and 3 in room B). At week 1, rectal swabs from 1-week-old piglets (two piglets per litter) were collected. At week 3, the same piglets were sampled again prior to weaning and being transferred to a different building. At week 8 (last week of the growing phase and prior to their move to the finishing building), rectal swabs were collected from 20 growers. Finally, at week 21, because the animals’ sizes and high concentrations in small pens prevented us from collecting individual samples, we collected fecal samples of 100 g from the floors of six pens. For each sampling, we took roughly 100 g from the UP of the rooms housing the sampled animals (room A at weeks 0, 1, and 3) as well as a sample from the FST (about 100 g). Finally, an additional sample of the FST was taken during week 53.

In summary, three sampling lines (animals, UP, and FST) were monitored to obtain 2 E. coli isolates from fecal samples from each individual animal and 20 E. coli isolates from each composite sample.

Detection and isolation of E. coli. One gram of each sample was suspended in 9 ml of distilled water and serially diluted 10-fold. We spread 0.1 ml from each dilution, including the original dilution, onto selective chromogenic medium (Coli ID; bioMérieux, Marcy-l’Etoile, France) to detect β-D-glucuronidase-producing E. coli colonies. After incubation (37°C for 24 h), the suspected colonies (2 or 20 colonies), based on their color and morphology, were subcultured on blood agar (bioMérieux, Marcy-l’Etoile, France) and incubated at 37°C for 24 h.

Identification of E. coli. E. coli was identified biochemically by the application of the IMVIC. The API20E system (bioMérieux, Marcy-l’Etoile, France) was applied to confirm the identification of E. coli isolates with unclear results (indole production, positive methyl red reaction, negative Voges-Proskauer test, and lack of ability to use citrate as the sole carbon source). Those isolates which remained unidentifiable were confirmed by a PCR technique (20), by using oligonucleotides derived from the uidA gene, encoding the β-D-glucuronidase enzyme.

Characterization of E. coli isolates by REP-PCR. E. coli DNA was extracted by the boiling method and stored at -40°C until its use. REP fingerprints were obtained by using PCR and oligonucleotides modified with inosine, as described previously by Vila et al. (21). Electrophoresis gels were normalized by using a standard molecular marker of 3,000 bp. Electrophoresis gel images were analyzed by using BioNumerics software (version 2.0; Applied Maths, Kortrijk, Belgium). Similarities among REP-PCR fingerprints were measured by using the Dice similarity coefficient, using a band-matching tolerance of 10%. The REP-PCR fingerprints (REP10) were then clustered by using the unweighted-pair group method with arithmetic means (UPGMA).

Antimicrobial susceptibility test. Susceptibility to 19 antimicrobials was determined according to the Spanish Veterinary Antimicrobial Resistance Surveillance (VAV) Network, as described previously by Teshager et al. (4). A microplate dilution method using a commercial microplate (Sensititre Salmonella plate-EUMVS; Trek Diagnostics System, East Grinstead, United Kingdom) was applied for determining the MICs of 13 antimicrobials using the following cutoff values for resistance: >64 mg/liter for sulfonamides (Su), >2 mg/liter for trimethoprim (Tm), >8 mg/liter for ampicillin (Am), >0.25 mg/liter for cefotaxime (Cr), >0.5 mg/liter for ceftazidime (Caz), >8 mg/liter for tetracycline (Te), >8 mg/liter for streptomycin (Sm), >2 mg/liter for gentamicin (Gm), >16 mg/liter for kanamycin (Ka), >16 mg/liter for chloramphenicol (Chl), >16 mg/liter for florfenicol (Fchl), >16 mg/liter for nalidixic acid (Na), and >0.06 mg/liter for ciprofloxacin (Cp). Moreover, an agar diffusion method was used to test the following six antimicrobials not included in the Sensititre plate: amoxicillin-clavulanic acid (Amc) (inhibition zone diameter resistance breakpoint of <14 mm), cefoxitin (Cfx) (<15 mm), imipenem (Imp) (<20 mm), aztreonam (Azm) (<18 mm), amikacin (Ak) (<15 mm), and apramycin (Apr) (<13 mm). Reference E. coli strain ATCC 25922 was included periodically as an internal control in the study.

Resistance cutoff values for Su, Tm, Am, Cr, Caz, Te, Sm, Gm, Cl, Fchl, Na, and Cp were those recommended by the European Union Reference Laboratory for Antimicrobial Resistance (22). Resistance breakpoints for Amc, Cfx, Ak, Ka (including intermediate isolates), Azm, and Imp were those recommended by the Clinical and Laboratory Standards Institute (23), whereas for Apr, we used the cutoff value recommended by the VAV Network (4).

DNA extraction and detection of integrons. E. coli DNA was extracted as mentioned above. A multiplex PCR was used to detect the int11, int12, and int13 genes, which encode class 1, 2, and 3 integrase, respectively, using primers described previously by Su et al. (24). For uncertain amplicon bands, single PCRs were performed to amplify the int11 and int12 genes (25). All the reactions were carried out in a final volume of 50 μl containing 10× standard buffer with MgCl2, 2.5 mM deoxynucleoside triphosphate (dNTP) mix (Biotools), 25 μM each primer, and 1.25 U DNA polymerase (Biotools), which included positive and negative E. coli control strains donated by the Universidad de La Rioja (Spain). The reaction conditions for the multiplex PCR were 1 cycle at 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min; on the other hand, the reaction conditions for the amplification of the int11 and int12 genes separately were 1 cycle at 94°C for 5 min followed by 30 cycles at 94°C for 5 min and 10 min, respectively, at 62°C and 55°C (30 s and 1 min, respectively) and 72°C for 1 min. In addition, we performed an amplification of the 3’ conserved segment (3’CS) (qaE1 and sull genes) in the sulfonamide-resistant int11-negative strains using primers described previously by Mazel et al. (25).

Population diversity studies. The E. coli population diversity of the 16 sampling groups was analyzed by considering two different typing approaches: a DNA-based (REP10) approach and a phenotypic (resistotype) approach. In both cases, population diversity was measured by using the Simpson diversity index (26) with confidence intervals (CI) according to those described previously by Grundmann et al. (27).

Population similarity studies. Population similarities between sampling groups in E. coli profiles based on both the REP10 and the resistotype data were analyzed by using a hierarchical cluster analysis method (IBM SPSS Statistics v. 19 software, 2010), after having previously transformed the quantitative data (number of isolates per typing group) on a scale ranging from 0 to 1, using the Ward method for clustering and the square Euclidean distance as a measure of distance.

Statistical methods. Chi-square tests were used to test differences among frequencies, and the Bonferroni correction was applied for multiple comparisons (WinPepi v11.22 software) (28); the Student t test was used for differences among means (IBM SPSS statistics v. 19 software, 2010).

RESULTS

Isolation and characterization of E. coli. A total of 475 E. coli isolates were confirmed, conventionally and/or with the help of the API20E system (10 isolates) or PCR (17 isolates).

In this study, the animal line was comprised of 256 isolates obtained from the five samplings of animals and the sampling of the finishers’ floor pen; 100 and 119 isolates were obtained from five and six samplings of the UP and the FST lines, respectively.

Characterization of the isolates by the REP-PCR technique. Twenty-five bands were detected, with sizes ranging from 235 to 2,487 bp. The number of bands among the individual isolates fluctuated between 0 and 13.

When the 475 isolates were clustered, 81 REP10 groups were identified, including 25 (31%) single groups (only one isolate per group) and 56 (69%) multiple groups (two or more isolates per group). Among the multiple groups, the largest ones were groups M (51 isolates), E (39 isolates), and R (36 isolates). Among the 56 multiple groups, 29% were detected in all three sampling lines, 41% were detected in two lines (20% in animals and FST, 16% in

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animals and UP, and 5% in FST and UP), 25% were detected in animals alone, 2% were detected in UP alone, and 4% were detected in FST alone. When the number of multiple-group isolates (n = 450) was considered, the figures were as follows: 55% were detected in all three sampling lines, 32% were detected in two lines (15% in animals and FST, 13% in animals and UP, and 4% in UP and FST), 12% were detected in animals alone, 1% were detected in FST alone, and 0.4% were detected in UP alone.

**Antimicrobial resistance.** Among the 475 isolates tested, we detected high levels of resistance to Te (86.5%), Su (80.0%), Sm (77.1%), Tm (67.4%), and Am (50.5%). There were low levels of resistance to Cl (17.5%), Cp (11.4%), Na (10.3%), Ka (8.2%), Gm (1.9%), Amc (1.7%), Cfx (0.6%), Fc (0.2%), and Apr (0.2%). Finally, all the isolates were susceptible to Cft, Caz, Imp, Azt, and Ak. The resistance percentages per sampling group are represented in Table 1.

Resistance profiles (resistotypes) were established by choosing the 14 antimicrobials in which at least one isolate was resistant. Eighty-six resistotypes were identified, including 42 (49%) single resistotypes (only one isolate per group) and 44 (51%) multiple resistotypes (two or more isolates per group). Of the multiple resistotypes, the most frequent ones were Te Su Tm Sm Am (90 isolates), Te Su Tm Sm (58 isolates), Te Su Tm Sm Am Cl (32 isolates), and Te Su Sm (31 isolates). Among the 44 multiple resistotypes, 32% were detected in all three sampling lines, 36% were detected in two lines (7% in animals and FST, 11% in animals and UP, and 18% in FST and UP), 20% were detected in animals alone, 5% were detected in UP alone, and 7% were detected in FST alone. When the number of isolates of multiple groups (n = 433) was considered, the figures were as follows: 75% were detected in all three sampling lines, 13% were detected in two lines (3% in animals and FST, 4% in animals and UP, and 6% in UP and FST), 9% were detected in animals alone, 2% were detected in FST alone, and 1% were detected in UP alone.

**Presence of integrons.** Sixty-nine percent (328 isolates) of the 475 isolates contained integrons: 37% (174 isolates) harbored class 1 integrons, and 40% (188) harbored class 2 integrons and any isolate class 3 integrins. Class 1 and 2 integrons were detected simultaneously in 7% (34) of the isolates. The frequencies of the presence of integrons per sampling group are represented in Table 2; these data showed that *E. coli* isolates from finishers have lower integron frequencies than isolates belonging to other animal groups.

**E. coli population diversity analysis.** Table 3 summarizes the values of the Simpson diversity index for the three lines studied according to the approaches mentioned above. Both approaches showed a high level of diversity in all the sampling groups, although the overlap among 95% CI values suggested no significant differences among them.

Temporal analysis of the diversity in the animal line showed some differences between the *E. coli* population of pregnant sows (diversity value of 0.968) and that of the 1-week-old piglets (0.854) based on REP10 fingerprints (Table 3). In addition, the diversity of the six finisher pen populations was disparate, irrespective of the approach.

**E. coli population similarity analysis.** Figure 1 shows the dendrogram obtained when the population diversity of the 16 sampling groups based on the REP-PCR profiles was analyzed. According to this clustering, finishers had an *E. coli* population well
TABLE 2 Percentages of integrons in the 475 porcine isolates belonging to different sampling groups and isolated in a farrow-to-finish farm.

<table>
<thead>
<tr>
<th>Sampling Group</th>
<th>% of integrons</th>
<th>% of int1</th>
<th>% of int2</th>
<th>% of both</th>
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<tbody>
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<td>Sows, wk 0</td>
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Note: Superscript letters indicate significant differences (Bonferroni corrected) between figures marked with the same letter on the same line (animals, UP, or FST).

DISCUSSION

Only a limited number of longitudinal studies performed in a single pig farm described _E. coli_ populations in animals and/or in the farm environment, with some of those studies being focused on antimicrobial resistance. Our results revealed that _E. coli_ populations in the pig fecal microbiota and in the farm environment are dynamic and show high levels of diversity. Some authors contended that animals harbor a limited number of resident _E. coli_ clones, but our data regarding diversity do not confirm these findings. Traditionally, it has been mentioned that individual-animal variation, the age of the animal, and characteristics of the intestine sections are the main factors responsible for the differences and flow changes in _E. coli_ intestinal populations in pigs. The data obtained showed that pregnant sows had different populations and higher levels of diversity than younger animals, as was mentioned in previous studies. When antimicrobial resistance profiles were compared, the examined sows and the 1-week-old piglets showed similar diversity levels but more distant populations. Although some authors detected higher levels of antimicrobial resis-
Diversity in intestinal E. coli population in a commercial pig farm

The diversity and clustering of E. coli isolates were assessed in a commercial pig farm using REP-PCR fingerprinting and resistotyping. The study included Finishers (3rd-9th week), Growers (10-18 weeks), and Sows (20-70 days). The diversity analysis showed a higher diversity in Finishers compared to Growers and Sows. The clustering analysis revealed distinct clusters for Finishers, Growers, and Sows, with Finishers showing a higher degree of diversity than Growers and Sows. The results indicate that the diversity and clustering of E. coli isolates in a commercial pig farm can be affected by the age and stage of pig development.
E. coli populations isolated from animals and manure in the same farm were compared previously (10, 11) in terms of similar methodologies (REP-PCR and the Simpson diversity index, among others), demonstrating that the population of stored manure was generally more diverse than that of fresh animal feces. This difference has not been detected in our study using REP-PCR typing; however, a slightly higher level of diversity in manure populations was observed when typing was based on resistotypes. The clustering of three out of the four FST samples taken during the first 8 weeks of the study suggests that a fraction of the manure E. coli population can persist for at least 2 months; however, resistotype-based clustering does not support this conclusion, indicating a different efflux of antimicrobial resistance traits.

The clustering of sampling groups based on REP-PCR typing showed that the spatial features (the line) weighed stronger than the temporal features (sampling week) in clustering, especially for environmental samples. This weight was less significant in clustering based on resistotypes. Viewing the results in their entirety, the environmental populations analyzed and the similarity between environmental populations analyzed and the similarity between animal and manure samples indicate that a different efflux of antimicrobial resistance traits exists.

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