Withdraw of prophylactic antimicrobials does not change resistome in pigs

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Research article

Keywords: Antimicrobial prophylaxis, pigs, resistome, antimicrobial resistance, mobile genetic elements, medicated feed

Posted Date: December 6th, 2019

DOI: <https://doi.org/10.21203/rs.2.18011/v1>

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Abstract

Background: The use of antimicrobials in the animal industry has increased the prevalence of antibiotic resistant bacteria and antimicrobial-resistance genes which can be transferred to human microbiota through the food chain or the environment. To reduce the influx of antibiotic-resistance to the human microbiota, restrictions on antimicrobials (in food animals) have been implemented in different countries. We investigated the impact of an antimicrobial restriction on the frequency of antimicrobial-resistant bacteria in pigs (PCI 1050) from an Ecuadorian farm.

Results: No differences in antimicrobial resistant coliforms or antimicrobial resistance genes (richness and abundance) were found when we compared animals fed with or without antibiotics. Nevertheless, the absence of antimicrobials in pigs didn't impact the productive performance of animals.

Conclusion: Fitness costs of antimicrobial resistance in bacteria within intestinal microbiota of animals seems to be overestimated. Avoiding antimicrobials as prophylactics in pigs fed is not enough to control maintenance and spread of antimicrobial resistance.

Background

Throughout history, antimicrobials have been effective in the treatment and control of bacterial diseases and have contributed to a greater life expectancy of humanity [1]. However, the constant use of antibiotics has created a selective force increasing the frequency of antimicrobial resistance genes (ARGs) associated with mobile genetic elements (MGEs) and their spread among bacterial populations [2, 3].

Around 75% of total antimicrobial production in the world is used in the food-animal industry [1] and, larger amounts of antibiotics may be used in animals in less industrialized countries which lack regulatory policies for antibiotic use [4, 5]. The use of antimicrobials in animals causes the proliferation of commensal bacteria with ARGs and resistance can be horizontally transferred to many other bacterial species in the intestines [6, 7].

Antimicrobial-resistant commensals from farm animals can end up in food products such as meat and dairy [1, 8–10]; these bacteria can colonize human intestines and could become opportunistic human pathogens. Also, ARG associated with MGE could be transferred from bacteria from food-animals to human microbiota [11, 12, 15–17]. For instance, *E. coli* strains isolated from healthy pigs carried an MGE with *mcr-1* gene which confers resistance to colistin [18] and the same MGE and gene has been found in commensal microbiota in humans and other animal species [19, 20]. This interaction between bacteria from food animals and humans has driven the creation of new policies and regulations aimed to reduce the use of antibiotics in farm animals [13, 14].

In theory, if ARGs cause fitness costs in commensal bacteria in the absence of antibiotics, therefore, the reduction in antibiotic use in farms should cause a reduction in antimicrobial-resistant bacteria (ARB)

food-animals [21]. However, the persistence of AR bacteria occurs thanks to compensatory evolution that allows bacteria to maintain ARGs without fitness costs [22, 23]. Experiments in which animals were deprived of antibiotics (as growth promoters) retained high levels of antibiotic resistance in numerically dominant *Escherichia coli* [24, 25] or high relative abundance of resistance genes [26]. More importantly, ARGs, multi-resistant and numerically dominant bacteria have been found in animals from organic farms [27–29]. The main objective of this study was to analyze the effect of the removal of antibiotics administered as prophylactics (higher antibiotic doses than for growth promotion) during 2 generations of animals. In this study we kept the animals receiving antibiotics close to those deprived of antibiotics to see if bacteria without ARGs can outcompete ARB for colonization in the absence of antibiotics.

Results

Based on coliform counts in MacConkey Lactose (MKL) culture media with and without antimicrobials, antimicrobial resistance ratios of resistant coliforms to overall coliforms were calculated and are shown in Table 2. No significant differences between treatment groups for any antibiotic was found ($p = 0.434$; 0.722 ; 0.763 respectively). The AR ratios for tetracycline were higher than those for trimethoprim-sulfamethoxazole or ampicillin.

Antimicrobial susceptibility tests for 537 randomly selected strains ($A = 266$ and $B = 271$) showed general resistance to ampicillin ($n = 397$; 73.9%), amoxicillin-clavulanic ac. ($n = 188$; 35%), tetracycline ($n = 434$; 81.1%), trimethoprim-sulfamethoxazole ($n = 301$; 56.1%), gentamycin ($n = 125$; 23.3%), ciprofloxacin ($n = 71$; 13.2%), chloramphenicol ($n = 174$; 32.4%) and ceftriaxone ($n = 77$; 14.3%) were detected. There were no significant differences ($\alpha \geq 0.05$) between treatment groups neither in sows nor in piglets (in nursing or fattening phases) (Table 3). Strains with resistance to 3 or more antimicrobials were considered as multidrug resistant phenotype (MDR) ($n = 354$; 65.9%).

The AR richness was not different ($\alpha \geq 0.05$) in animals within-group neither between groups (Figure 3) and the abundance of ARGs decreased over time, although tetracycline resistance genes and MGE remained stable. Among the most abundant genes detected were aminoglycoside resistance and MGEs: *Tp614*, *IS613*, *tnpA*, *int1-a-marko*, *int2*, *int11F165_clinical*, *pBS228-lncP-1*, *trb-C*, *IS26*, *IS256*, *IS6100*, *IS91*. Some of these MGEs could be responsible for the transference of resistance genes among microbiota species. Genes such as *tet* (32) was detected in all samples and colistin resistant gene presence was low and was reported in the category “other”. A PCR amplification of *mcr-1* gene in fecal samples showed that 19 of the 20 sows carried bacteria with this gene.

A Spearman correlation test was performed on QIUcore Omics Explore 3.4 software showed no difference of ARG relative abundance profiles between samples collected during different growth phases. Pigs at day 30, showed a higher ARG relative abundance (although not it is not statistically significant) than pigs on day 5, however, this high ARG relative abundance declined over time (Figure 3). There were no statistic differences in weigh, growth and health status among study groups (S2 supplementary materials).

Discussion

In this study, we used a commercial farm context to analyze the impact of the antimicrobial restriction on antibiotic resistance in coliforms and microbiota along the life period of 2 generations of pigs. We expected that the absence of prophylactic doses of antimicrobials in the diet will cause antimicrobial sensible bacteria to outgrow resistant ones [30, 31]. However, we did not find significant differences ($p \geq 0.05$) in the total number of resistant coliforms nor did we find differences in resistance genes (abundance or diversity) between groups of animals receiving and those deprived of antibiotics (Figures 2, 3 and 4). We also failed to detect any differences in the microbiota resistome (amount and type of ARGs) between the two groups of animals. These results are in agreement with other reports showing no effect of antibiotic restriction on the proportion of ARB of animals [32]. Similar AR phenotypes have been found in isolates from animals with and without antimicrobial restrictions (conventional vs. organic farms) [27, 31, 33, 34]. Contrary, other studies showed an important decrease in resistance bacterial isolates or resistant gene abundance after antimicrobial removal [25, 29, 35].

Our results may indicate that ARGs are not causing a major fitness reduction in the bacterial population from pig intestines. Under laboratory conditions, it has been shown that the fitness costs associated with a plasmid carriage disappear and even could transform in fitness advantage after 420 generations [36].

Furthermore, this advantage could even be transmitted to other bacterial hosts never exposed to this plasmid [36]. Under farm conditions, due to a long period of antimicrobial feed supplementation, coliforms could be well adapted to permanent antimicrobial concentrations and the reversibility of antimicrobial resistance in the intestine could be slow or antibiotic-sensible bacteria could not outcompete resistant ones [23].

Antimicrobial susceptibility test showed a higher resistance frequency to tetracycline, ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol without differences among groups; these resistances are common in pig farms [29]. The resistance genes with higher relative abundance were tetracycline, lactams, and aminoglycosides (Figure 3). Genes associated with tetracycline resistance have been detected in pigs feeding with or without medicated feed which concurs with the notion tetracycline resistance genes are also common in pig's intestinal resistome [25, 35, 37, 38]. The presence of lactam or aminoglycoside resistance genes occurred despite these antimicrobials were not used as growth promotor, prophylactics or therapeutics; similar observations have been reported previously [35, 39].

These observations suggest that antibiotic restriction was not enough to reduce microbiota antibiotic resistance in this pig farm. It is possible that AR bacteria present in the surrounding environment colonized successfully the intestines of animals deprived of antibiotics. Reducing antibiotic resistance in these environments may require antibiotic restriction in the totality of animals and for longer periods [26]. The resilience of antibiotic resistance in the microbiota is an important factor against the reduction of ARB transmitted to humans [40].

MGEs are important actors in antimicrobial resistance spread [41] and in this study we observed a higher relative abundance of MGEs in samples in 30 day-old pigs (Figure 3), corresponding to the weaning period which may affect the gut microbiome [42]; this transition may cause a dysbiosis (from milk feeding to solid feed) [43, 44]. Bacteria under stress may turn on their S. O.S response which increases mobilization of MGEs and subsequent increase in numbers [45, 46]. The mobile genetic elements markers, that showed an important relative abundance, may explain the abundance of ARGs [39].

Housing the two groups of animals in the same barn may be an important factor contributing to the similarity of AR in bacteria from both groups. However, we hypothesized that microbiota of animals without antibiotics should be re-colonized by antimicrobial susceptible bacteria which should be able to outcompete AR bacteria in the surrounding environment. Finally, the withdraw of antibiotics did not have any repercussions in the growth of the animals.

Conclusions

Our observations suggest that antibiotic restriction is not enough to reduce the numbers of antibiotic-resistant bacteria in the gastrointestinal tract of food animals and their products. The maintenance of antibiotic resistance in the absence of antibiotic pressure is not easily explained, there are many evolutionary factors that are not fully understood and require additional research.

Abbreviations

AR.- Antimicrobial resistance

ARB. - Antimicrobial resistant bacteria

ARG. - Antimicrobial resistance gene

ARGs.—Antimicrobial resistance genes

MGE. - Mobile genetic element

MGEs. - Mobile genetic elements

MDR. - Multidrug resistance

MKL. - Mac Conkey Lactose

n.- number of samples

PCR.—Polymerase chain reaction

SXT. - Trimethoprim and 76 mg /liter of sulfamethoxazole

CFU.—Colony forming units

PO. - phosphate-buffered saline solution

TSB. - tryptic soy broth

mL.- milliliter

Methods

All protocols of experimental design were approved by the ethics and biosecurity committee of the company and the Animals Ethics Committee of Universidad San Francisco de Quito before the study.

Animals

A completely random, balanced fixed-effects double-blind study was conducted in two generations of pigs housed in a farm in Ecuador. The number of animals was calculated with a statistic power test of 87% considering the probable loss of experimental units (replacement sows). The study was conducted under strict biosecurity conditions. Twenty 70-day female PCI 1050 pigs were randomly assigned to each of the 2 groups. Sows were raised in a Site 1 farm where they were inseminated and carried out their pregnancies. One group containing 10 female pigs (the control group) was fed with conventional feed formulation with antimicrobial additives (group A) and, and the other comprised 10 female pigs which were fed without antimicrobial additives in the feed (group B).

Treatments were maintained during the whole study period (S1, supplementary materials). Three sows from treatment A and 4 sows from treatment B farrowed at the same time (there were no differences in litter numbers between treatments) (S2. Supplementary materials), piglets were placed with their corresponding mothers. When piglets reached 20 days, they were weaned and housed in another facility where they were distributed into two separate pens for group A (n = 40) and B (n = 40) and continued with treatments of their respective mothers. Each piglet was identified with a numbered earring. Once piglets were 70 days, they were moved to site 3 for fattening (71d to 170 d) (S2, supplementary materials); animals were kept under the same treatment throughout this time. The productive performance was observed based on dairy weight gain along the study period and after slaughtering the carcasses weight was compared. Vaccines were administered to all animals and the antimicrobial treatment was administered under veterinarian supervision only to animals that have any diagnosed infection. Antimicrobial additives used are described in Table 1.

During the weaning and fattening phases, each pen grouped 32 piglets. Pig density was 0,45 m²/pig in the weaning phase and 0,90–1,0m²/pig at the fattening phase. Animals from each group were monitored for 150 days (Figure 1). The type and antimicrobial concentrations in feed changed over time and have

been used routinely in the farm for the two previous years (Table 1). Animals were released from the experiment at day 150 and they were slaughtered (electric stunning) at day 170.

Housing and husbandry

The maternity and weaning phases (piglets until 70 days) are carried out in pens with plastic slatted flooring, the pens for fattening phase were located in a different facility and had cement floor which was washed daily. In all growing phases, pens were protected from the sun with roof, ceiling was at 2,5m high. Number of cages are described in Fig, 1.

Tap water and feed were administered *at libitum*, temperature ranges from 20 to 26°C. All experiments were performed under veterinary supervision. If any sick animal was detected it was immediately spared in a different cage to be treated and eliminated from the study.

Samples and phenotypic analysis

Two Rectal swabs were taken from each sow and a randomly selected piglet from each pen during one productive cycle (Figure 1). Swabs were maintained on ice for transportation to the lab facilities within 2h after collection. Intestinal coliforms were used as a microbial indicator of phenotypic resistance.

For quantification of coliform resistant bacteria in intestinal microbiota of pigs, MKL medium was supplemented with 4 mg / liter of trimethoprim and 76 mg /liter of sulfamethoxazole (SXT) [50], or 32 mg/liter of tetracycline [51], or 16mg/liter of ampicillin according to protocols described previously [52].

Swabs were eluted in 5mL of sterile tryptic soy broth (TSB) diluted in phosphate-buffered saline solution (PO) (0.0169M KH_2PO_4 , 0.0719M K_2HPO_4 , pH 7,2) [51]. A 0.1mL aliquot of this solution was serially diluted in 0.9 mL of PO until 10^{-3} and 0.1mL of each dilution was plated onto the surface of MKL plates with and without antimicrobials [52] (S3. Supplemented materials). We estimated the ratio of resistant coliforms by counting the number of colonies in MKL plates with antimicrobials divided by the colonies in MKL plate without antimicrobials [29]. Only plates with coliform counts greater than 4 colony forming units (CFU) were recorded (S3, supplementary materials).

Antimicrobial susceptibility test

One lactose fermenting (coliform) colony from each plate was isolated and stored at -80°C in TSB with 30% glycerol [34]. Antimicrobial susceptibility tests were performed using AMP ampicillin (10g), TET tetracycline (30g), SXT trimethoprim-sulfamethoxazole (1.25/23.75g), GEN gentamycin (10g), AMC amoxicillin-clavulanic ac. (20/10g), CIP ciprofloxacin (5g), CHLOR chloramphenicol (30g) and COX ceftriaxone (30g) as representatives of the most used families of antibacterial drugs used in health care

[53, 54]. The Kirby Bauer test was carried out following CLSI (Clinical & Laboratory Standards Institute) guidelines.

Molecular analysis

For molecular analysis, swabs were frozen at -80°C until they were used. DNA from swabs taken for each pig was isolated using MO BIO Power Soil DNA Isolation Kit (MO BIO, 12888–100) using swab dilution material in bead solution buffer and following manufacturer instructions. Quality and quantity were evaluated using nanodrop (Thermo Scientific) and Qubit dsDNA HS (Thermo Fisher Scientific, Oregon, USA). From sows' samples, *mcr-1* PCR amplification was performed used the conditions described previously [18]. One fecal pooled sample, from each sampling point (6 from A and 6 from B group) was analyzed in duplicate with high throughput qPCR. WaferGen SmartChip Real-time PCR system was used to detect 384 genes, 338 are informative for AR genes or MGE. Primers for these genes and associated HT- qPCR assay were designed, used, and validated in the previous studies [35, 55–57], and primer set was update recently [58] (Suppl. 1). The genetic richness was defined as the number of AR genes found in a niche.

Statistical analysis

All collected data were registered in MS EXCEL software and descriptive and inferential statistical analysis were performed in INFOSTAT (Statistic Software Vs 2017). The impact of the antimicrobial restriction on coliforms count, on the susceptibility patterns of isolates, and animal performance was compared with T-test and Chi-Square respectively (≤ 0.05). HT-qPCR data were analyzed according to previously established methods [35, 59]. Specifically, $\Delta\Delta\text{CT}$ method was used to normalize and calculate the fold change. Moreover, the relative abundance of ARGs was calculated with normalization to the universal 16S rRNA; estimated from the Ct value with a conservative threshold Ct of 30 as the gene copy detection limit. Calculated data represent the copy number per 16S rRNA gene copy. QIUcore Omics Explore 3.4 software was used to show heat maps.

Declarations

Ethics approval and consent to participate

All the experimental procedures were approved by the Animal Ethics Committee at Universidad San Francisco de Quito document Nr. 2016–004. A technical committee in the company provided their approval for this study (document number Cn.Ce.Ca.16.04). Company's consent was implicit as the protocols were designed jointly between the researchers and Company's technical personnel; this agreement was confirmed in a consent letter sent from the Company to the Animal Ethics Committee at Universidad San Francisco de Quito. was required. The number of animals under research was approved by farm managers. All facility adequation and sampling methods were performed under farm's

veterinarian supervision. Approval of all the protocols and approval publications were obtained from the technical managers of the company. The name of the company was kept confidential at the company's request. There is no national requirement to obtain IRB approval for this type of studies.

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author under any request

Competing interests

The research was conducted without any potential conflict of interest.

Funding

This project was funded by Universidad San Francisco de Quito Grant No. 11182. USFQ and the Company (we weren't authorized to disclose the name of the company).

Author contributions

GT Funding acquisition, Farm access agreement, and project conceptualization

FL: Experimental design, field and lab techniques, sampling procedures, data register and analysis, write and edition of the publication.

AT: experimental design, farm permissions, and animal welfare.

LZ Molecular methodology, technical support in high throughput qPCR. WaferGen SmartChip Real-time PCR system and data analysis.

LZ and GT manuscript edition

All authors have read and approved the manuscript.

Acknowledgments

We thank Germán Romo, Manuel Salgado, Gustavo Nava, and Edgar Velarde for their technical advice.

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Tables

Table 1. Antimicrobial additives used in pigs farm as prophylactics in group A.

Growth phase	Age (days)	Antimicrobial	Dose, ppm	Administration via
0	21 - 28	Tilmicosin	200	Food
		Colistin	40	Food
1	29 - 34	Tiamulin	150	Food
		Chlortetracycline	450	Food
2	35 - 45	Tiamulin	150	Food
		Chlortetracycline	450	Food
3	45 - 70	Tiamulin	150	Food
		Chlortetracycline	450	Food
4	70 - 85	Chlortetracycline	450	Food
5	123 - 139	Chlortetracycline	450	Food

2	37 - 40	Trimetoprim-sulfamethoxazole	25mg/Kg/PV	Water
3	45 - 47	Doxicycline	10mg/Kg/PV	Water

Table 2. Antimicrobials used as supplements to MacConkey Lactose (MKL) culture media.

Antimicrobial		Concentration	Reference
Tetracycline	TET	32mg/L	(Agga et al., 2016)
Ampicillin	AMP	16 mg/L	(Bibbal et al., 2007; Havelaar et al., 1987)
Trimethoprim-Sulfamethoxazole	SXT	4mg/L 76mg/L	(Schmidt et al., 2015)

Table 3. Total count of coliform colony forming units (CFU) in Mac Conkey lactose without antimicrobials. The average and standard deviation (SD) is shown for each treatment. Antimicrobial resistance ratios for ampicillin (AMP), cotrimoxazole (SXT) and Tetracycline (TET) were calculate using the total count of coliform colony forming units in Mac Conkey Lactosa plates with antimicrobials divided by the total count of coliform in Mac Conkey Lactosa without antimicrobials.

	Treatment A					Treatment B				
AGE (days)	TOTAL COUNT (AVERAGE)	SD	AMP	SXT	TET	TOTAL COUNT (AVERAGE)	SD	AMP	SXT	TET
5	1,73E+07	1,15E+07	0,48	1,22	2,15	2,34E+07	1,10E+13	0,39	1,55	2,38
30	6,71E+05	1,01E+06	0,78	0,56	0,66	5,15E+04	7,92E+14	0,98	0,43	0,97
50	9,17E+05	8,70E+05	0,26	0,48	0,83	8,90E+05	8,41E+14	0,48	0,48	1,01
100	2,46E+05	1,68E+05	0,38	0,94	0,99	7,40E+04	1,16E+13	0,48	1,48	0,72
140	1,08E+05	1,68E+05	0,34	0,32	1,05	2,65E+05	2,55E+14	0,25	0,14	1,2

Figures

Figure 1

Workflow for a productive pig's cycle of each treatment group (A and B). Treatment group A. were fed with antimicrobial supplements. Treatment group B were fed without antimicrobial supplements. Ten young female pigs (70d) were randomly selected for each treatment. All farrowed piglets were maintained under the same treatment group until day 2 (breastfeeding phase). A homogeneous group of 32 piglets (similar age and weight) within each treatment group were selected to form a weaning phase pen (since

22 days to 70 days) that was conserve until the slaughter phase (170 days). Rectal swabs samples were collected at 1.- 5 days; 2.- 30 days; 3.- 50 days: 4.- 100 days. 5.- 140 days. Samples from sows were taken at 180 days (6).

Figure 2

Antimicrobial resistance genes (ARGs) richness. Number of genes of genes of each class of antimicrobial target. In columns there are assigned the growing phase of piglets (1.- 5 days; 2.- 30 days; 3.- 50 days: 4.- 100 days. 5.- 140 days) and sows (6.- 180 days). Animals feeding antimicrobials were identified as A and animal without antimicrobial additives were identified with B.

Figure 3

Relative abundance (RA%) of antimicrobial resistance genes, grouped by sampling phase and treatment group (A.- Pigs fed antimicrobials; B.- pigs without antimicrobials in feed; 1.- 5 days; 2.- 30 days; 3.- 50 days; 4.- 100 days; 5.- 140 days) and sows (6.- 180 days).

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