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Escherichia coli used as a biomarker of antimicrobial resistance in pig farms of Southern Brazil

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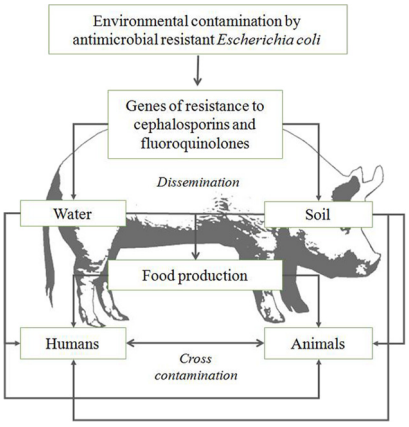
HIGHLIGHTS

- Prevalence of *E. coli* in fecal, water and soil samples of swine farms from Southern Brazil
- Phenotypic profile of resistance and multiresistance of strains of *E. coli* to five classes of antimicrobials
- Detection of Extended Spectrum Beta-lactamases (ESBLs) in *E. coli* strains
- Coexistence of ESBLs and *qnr* genes in phenotypically ESBL-producing isolates

GRAPHICAL ABSTRACT



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ABSTRACT

The objective of this study was to verify the presence of antimicrobial resistant strains of *Escherichia coli* in pig farms and to use it as a biomarker to evaluate phenotypic and genotypic profiles of antimicrobial susceptibility, as well as the presence of Extended Spectrum Beta-lactamases (ESBLs) and fluoroquinolone resistance genes. Several samples (n = 306) collected from swine farms (n = 100) of Southern Brazil were used for *E. coli* isolation: 103 of swine feces, 105 of water, and 98 of soil. *E. coli* isolates were submitted to the disk-diffusion test to verify their antimicrobial susceptibility, to disk-approximation test to detect ESBL-producers, and to PCR analysis to search for ESBLs genes (*bla*CTY-M2, *bla*SHV-1, *bla*TEM-1, *bla*CTX-M2, *bla*OXA-1, *bla*PSE-1) and quinolone resistance genes (*qnr*A, *qnr*B and *qnr*S). The percentage of *E. coli* isolates found in feces, water and soil samples was 66.02%, 30.48% and 35.71%, respectively. The highest percentages of resistance were obtained for sulfamethoxazole associated with trimethoprim (63.70%), colistin (45.19%) and enrofloxacin (39.26%). Regarding the levels of multidrug resistance, 37.04% of the isolates were resistant to three or more classes of antimicrobials. The most common profile (16%) of multirresistance was GEM-SUT-ENO-COL. The index of multiple resistance to antimicrobials (IRMA) was above 0.2 in 78% of the multiresistant isolates. Out of 135 *E. coli* isolates, 7.41% was ESBL-producers, of which 50% showed the *bla*CMY-M2 gene, 40% the *bla*TEM-1 and 70% the *qnr*S gene. Of non-ESBL-producing strains resistant to enrofloxacin, 13.04% were positives for *qnr*S gene. These results demonstrated

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the presence of fecal contamination in the environment, in addition to high resistance indexes for several antimicrobials, including beta-lactams and fluoroquinolones, which was confirmed by the genetic detection of ESBLs and *qnr* genes.

1. Introduction

Brazil is one of the largest animal protein producer, with 3.3 million tons of pork meat produced in 2017, where 600 thousand tons were exported to more than 70 different countries (ABPA, 2017). In 2016, the state of Santa Catarina (Southern Brazil) accounted for 38% of all exported Brazilian pork meat (EPAGRI, 2017). However, high concentration of animal production can lead to environmental problems, such as soil saturation caused by animal waste and also soil and water contamination by pathogenic microorganisms (Silva et al., 2015).

Contaminated water and soil may spread pathogenic microorganisms, and the bacterium *Escherichia coli* can be used as a biomarker of environmental contamination. *E. coli* is a commensal microorganism of the gastrointestinal tract of humans and many animals that can cause diseases and the spread antimicrobial resistance genes (Caldorin et al., 2013). It is indicated as a biomarker due to its abundance and direct contact with the gastrointestinal tract of animals, being susceptible to all environmental conditions of this organ, such as feed, additives and performance enhancers (Rochelle-Newall et al., 2015).

Recently, bacteria isolated from many environments have been identified as important reservoirs of resistance genes that can be transferred to other pathogens through mobile elements, such as genes that encode the production of Extended Spectrum Beta-lactamases (ESBLs), confering resistance to beta-lactam antibiotics (Jiang et al., 2011). These same mobile elements confer resistance to quinolones and fluoroquinolones through genes encoding the production of proteins capable of protecting the bacterium from drug action (Yeh et al., 2017).

Cephalosporins and fluoroquinolones are widely used in human and animal medicine and are classified as "critically important antimicrobials" by the World Health Organization (WHO, 2014). Although Santa Catarina State has been a state with a large pork meat production for many decades, little is known regarding its antimicrobial resistance status. In this sense, the objective of this work was to isolate *E. coli* from feces, water and soil of farms with swine production in order to evaluate its phenotypic profile of antimicrobial susceptibility, as well as to correlate the presence of ESBLs and *qnr* genes in beta-lactam and fluoroquinolone resistant strains of *E. coli*.

2. Materials and methods

2.1. Sampling and E. coli isolation

A total of 306 samples were collected from March 2016 to May 2017 (103 samples of swine feces, 105 of water and 98 samples of soil) from rural farms (n = 100) with pork production in many cities (n = 20) of Santa Catarina state, mainly in the municipalities of Seara and Xavantina, where the highest volume of meat production takes place (EPAGRI, 2017). The identification and origin of the samples are described in Tables 3, 4 and 5. The samples were conditioned in sterilized bottles and transported under refrigeration to the laboratory. *E.coli* isolation was performed according to the technique of Quinn et al. (2005) with some adaptations, where the samples were incubated in lactosate broth (1:10) for 24 h at 36 ± 1 °C. They were then seeded in petri dishes containing Eosin Methylene Blue Agar (EMB) and MacConkey Agar and incubated at 36 ± 1 °C for 24 h. The colonies with green metallic characteristics on EMB Agar and pink on MacConkey Agar were submitted to biochemical tests (Urea Base Agar, TSI Agar, Sim Medium Agar and Agar Simmons Citrate) and subsequently incubated at 36 ± 1 °C for 24 h. After biochemical confirmation of the colony as *E. coli*, it was incubated in microtubes containing Tryptone Soya Agar medium at 36 ± 1 °C for 24 h and stored −20 °C after the addition of steril glycerol.

2.2. Antimicrobial susceptibility test

For the antimicrobial susceptibility test, the methodology approved by the Clinical and Laboratory Standards Institute (CLSI, 2018) and the National Agency for Sanitary Surveillance (ANVISA, 2003) was used, which is included in the Normative Instruction M-2 A-8 Antimicrobial Susceptibility by Disk Diffusion, where five classes of antimicrobials (ATBs) with different mechanisms of action were tested, with the selected antimicrobials commonly used in swine production in Brazil: beta-lactams (amoxicillin associated with clavulanic acid 30 μg - AMC) and third-generation cephalosporin (ceftiofur 30 μg - CTF), fluoroquinolones (enrofloxacin 5 μg - ENO), aminoglycosides (gentamicin 10 μg - GEM), sulfonamides (trimethoprim associated with sulfamethoxazole 25 μg - SUT) and polymyxins (colistin 10 μg - COL) by

Table 1
Genes of resistance related to extended-spectrum beta-lactamases (ESBLs), primers, conditions for their amplification and sizes of each expected fragment (bp).

Gene	Nucleotide sequence	PCR conditions	Cycles	bp
*bla*CMY-2	(F) TGG CCG TTG CCG TTA TCT AC (R) CCC GTT TTA TGC ACC CAT GA	95 °C - 10 min; 95 °C - 30 s; 55 °C - 1 min; 72 °C - 1 min; 72 °C - 7 min; 4 °C - ∞		870
*bla*SHV-1	(F) GGC CGC GTA GGC ATG ATA GA (R) CCC GGC GAT TTG CTG ATT TC			714
*bla*TEM-1	(F) CAG CGG TAA GAT CCT TGA GA (R) ACT CCC CGT CGT GTA GAT AA			643
*bla*CTX-M2	(F) GGC GTT GCG CTG ATT AAC AC (R) TTG CCC TTA AGC CAC GTC AC		30	486
*bla*OXA-1	(F) AAT GGC ACC AGA TTC AAC TT (R) CTT GGC TTT TAT GCT TGA TG			595
*bla*PSE-1	(F) TGC TTC GCA ACT ATG ACT AC (R) AGC CTG TGT TTG AGC TAG AT			438
AmpC	(F) AAC ACA CTG ATT GCG TCT GAC (R) CTG GGC CTC ATC GTC AGT TA	95 °C – 9.5 min; 95 °C – 45 s; 59 °C - 45 s; 72 °C - 1 min; 72 °C - 7 min; 4 °C - ∞	40	1226

Table 2
Genes of the *qnr* family related to fluoroquinolone resistance, primers, conditions for their amplification and expected fragment sizes (bp).

Gene	Nucleotide sequence	Conditions for PCR	Cycles	bp
*qnr*A	(F) TCAGCAAGAGGATTTCTCA (R) GGCAGCACTATTACTCCCA	94 °C – 5 min; 94 °C - 45 s; 48 °C - 45 s; 72 °C – 1 min; 72 °C – 5 min; 4 °C - ∞	32	627
*qnr*B	(F) GATCGTGAAAGCCAGAAAGG (R) ACGATGCCTGGTAGTTGTCC	94 °C – 5 min; 94 °C - 45 s; 53 °C - 45 s; 72 °C – 1 min; 72 °C – 5 min; 4 °C - ∞	32	469
*qnr*S	(F) ACGACATTCGTCAACTGCAA (R) TAAATTGGCACCCTGTAGGC			417

the company DME® (Specialized Microbiological Diagnostics). After 24 h of incubation, the halo of bacterial growth inhibition was measured, and the bacterium was classified according to the tables described by CLSI (2018) as resistant, intermediate or susceptible. A sample of *E. coli* ATCCA® 25922 was used as control.

2.3. Multiple Drug Resistance (MDR) and Multiple Antimicrobial Resistance Index (IRMA)

The most prevalent multidrug resistance (MDR) profiles were also observed from disk-diffusion data, where it was possible to determine the number of *E. coli* isolates that were considered multiresistant (concomitant resistance to three or more classes of antimicrobials) (Frye and Cray, 2007). The Multiple Antimicrobial Resistance Index (IRMA) was calculated according to the methodology described by Krumperman (1983), which shows the relationship between the number of resistant antimicrobials and the total number of classes tested. IRMA can be used to identify the risk of transmission to humans, where strains with values above 0.2 are considered of high risk.

2.4. Disk-approach test for detection of ESBLs

The disk-approximation test was performed to detect ESBL-producers according to Jarlier et al. (1988). *E. coli* was inoculated into a tube containing 3 ml of lactose broth and incubated at 37 °C ± 1 for 8 h or until the turbidity level reached 0.5 on the McFarland scale. Using a sterile swab moistened in the bacterial suspension, the sample was inoculated smoothly in all directions of the plate containing Muller-Hinton agar, and with the aid of a flared and cooled clamp, the disks containing each antimicrobial were placed between a distance of 25 mm. The disks used for this test were: clavulanic acid-amoxicillin (AMC) (30 μg) in the center of the plate to visualize the synergism of the beta-lactamase inhibitor clavulanic acid with the other antimicrobials: azetreonam (ATM) (30 μg); ceftazidime (CAZ) (30 μg); ceftriaxone (CRO) (30 μg) and cefepime (CPM) (30 μg), were subsequently incubated at 37 °C ± 1 for 24 h. A sample of *E. coli* ATCC® 25922 was used as control. After incubation, strains positives for ESBLs were verified by the presence of a "ghost zone", represented by the enlargement of the zone of inhibition between the disks used.

2.5. DNA extraction and amplification by Polymerase Chain Reaction (PCR) to search for genes related to the production of ESBLs and resistance to fluoroquinolones

For the detection of resistance genes related to beta-lactams, DNA was extracted from *E. coli* ESBL-producers using the PureLink® Genomic DNA for Purification of Genomic DNA kit (Invitrogen, Life Technologies, Carlsbad). PCR was performed for the following ESBLs genes: *bla*CMY-M2, *bla*SHV-1, *bla*TEM-1, *bla*CTX-M2, *bla*OXA-1, *bla*PSE-1 and *Amp*C using a BioRad brand T100TM Thermal Cycler® equipment. Reactions were performed as recommended by Platinum® PCR SuperMix (Invitrogen, Life Technologies, Carlsbad, USA). The primers used and the conditions for amplification of these genes were used according to Chen et al. (2004) and conditions for amplification of the *Amp*C gene were performed according to Alcaine et al. (2010) (Table 1).

Due to the fact that ESBLs genes are usually in the same plasmid that confers resistance to quinolones and the later be easily spread throughout the environment, we searched for *qnr*A, *qnr*B and *qnr*S genes in *E. coli* isolates resistant to enrofloxacin and ESBL-producers (Moumoni et al., 2017). The primers used (Yue et al., 2008), as well as PCR conditions are described in Table 2. These genes were selected due to the great use of related antimicrobials (fluoroquinolones and beta-lactams) and the coexistence of these genes in the same plasmids, facilitating the dispersion of these genes among bacterial strains (Yue et al., 2008).

As a positive control for the PCR reaction, a *Salmonella* Heidelberg sample was used for amplification of the *inv*A gene previously detected, where the amplification conditions were obtained as described by Singh

Table 3
Description of swine fecal samples collected for *E. coli* isolation from swine farms of Southern Brazil and their antimicrobial resistance profile.

City	Source samples	N° Samples	N° Positives	Beta-lactams		Aminoglycosides	Sulfonamides	Fluoroquinolones	Polymixins	MDR[a]
				AMC	CTF	GEM	SUT	ENO	COL	
Seara	Nursery	8 (7.77%)	6 (5.83%)	1 (1.47%)	0	1 (1.47%)	4 (5.88%)	4 (5.88%)	4 (5.88%)	2 (2.94%)
	Termination	9 (8.74%)	8 (7.77%)	1 (1.47%)	2 (2.94%)	2 (2.94%)	8 (11.76%)	5 (7.35%)	3 (4.41%)	4 (5.88%)
	Gestation	15 (14.56%)	11 (10.69%)	1 (1.47%)	5 (7.35%)	5 (7.35%)	8 (11.76%)	4 (5.88%)	6 (8.82%)	5 (7.35%)
	Maternity	19 (18.45%)	16 (15.54%)	4 (5.88%)	5 (7.35%)	6 (8.82%)	11 (16.19%)	9 (13.24%)	7 (10.29%)	10 (14.71%)
Total		**51 (49.52%)**	**41 (39.83%)**	**7 (10.29%)**	**12 (17.65%)**	**14 (20.59%)**	**31 (45.59%)**	**22 (32.35%)**	**20 (29.41%)**	**21 (30.88%)**
Xavantina	Nursery	4 (3.88%)	3 (2.91%)	0	2 (2.94%)	1 (1.47%)	3 (4.41%)	3 (4.41%)	3 (4.41%)	3 (4.41%)
	Termination	12 (11.65%)	4 (3.87%)	0	0	0	2 (2.94%)	1 (1.47%)	1 (1.47%)	0
	Gestation	9 (8.74%)	5 (4.86%)	0	0	1 (1.47%)	3 (4.41%)	3 (4.41%)	0	0
	Maternity	12 (11.65%)	8 (7.77%)	0	1 (1.47%)	2 (2.94%)	6 (8.82%)	3 (4.41%)	2 (2.94%)	2 (2.94%)
Total		**37 (35.92%)**	**20 (19.43%)**	**0**	**3 (4.41%)**	**4 (5.88%)**	**14 (20.58%)**	**10 (14.70%)**	**6 (8.82%)**	**5 (7.35%)**
Other cities	Nursery	0	0	0	0	0	0	0	0	0
	Termination	12 (11.65%)	4 (3.87%)	1 (1.47%)	0	3 (4.41%)	4 (5.88%)	3 (4.41%)	1 (1.47%)	3 (4.41%)
	Gestation	2 (1.94%)	2 (1.94%)	0	2 (2.94%)	1 (1.47%)	2 (2.94%)	2 (2.94%)	1 (1.47%)	2 (2.94%)
	Maternity	1 (0.97%)	1 (0.97%)	0	0	0	0	0	1 (1.47%)	0
Total		**15 (14.56%)**	**7 (6.80%)**	**1 (1.47%)**	**2 (2.94%)**	**4 (5.88%)**	**6 (8.82%)**	**5 (7.35%)**	**3 (4.41%)**	**5 (7.35%)**
Grand total		**103 (100%)**	**68 (66.02%)**	**8 (11.76%)**	**17 (25%)**	**22 (32.35%)**	**51 (75%)**	**37 (54.41%)**	**29 (42.65%)**	**31 (45.59%)**

Beta-lactams (amoxicillin associated with clavulanic acid - AMC) and third generation cephalosporin (ceftiofur - CTF); Fluoroquinolones (enrofloxacin - ENO); Aminoglycosides (gentamicin - GEM); Sulfonamides (trimethoprim associated to sulfamethoxazole - SUT) and polymyxins (colistin - COL).

[a] Resistance to more than 3 classes.

Table 4
Description of water samples collected for *E. coli* isolation from swine farms of Southern Brazil and their antimicrobial resistance profile.

Cities	Source samples	N° Samples	N° Positives	Beta-lactams		Aminoglycosides	Sulfonamides	Fluoroquinolones	Polymixins	MDR*
				AMC	CTF	GEM	SUT	ENO	COL	
Seara	Well	3 (2.86%)	1 (0.95%)	0	0	1 (3.13%)	0	0	0	0
	Pond	1 (0.95%)	0	0	0	0	0	0	0	0
	Drinkers	1 (0.95%)	0	0	0	0	0	0	0	0
	Font	11 (10.48%)	5 (4.76%)	0	0	2 (6.25%)	1 (3.13%)	2 (6.25%)	3 (9.38%)	2 (6.25%)
	River	2 (1.90%)	2 (1.90%)	0	0	0	1 (3.13%)	1 (3.13%)	0	0
	Artesian well	13 (12.38%)	3 (2.86%)	0	1 (3.13%)	1 (3.13%)	1 (3.13%)	1 (3.13%)	2 (6.25%)	1 (3.13%)
	Caxambu font	2 (1.90%)	0	0	0	0	0	0	0	0
	Cistern	2 (1.90%)	0	0	0	0	0	0	0	0
Total		**35 (33.33%)**	**11 (10.48%)**	**0**	**1 (3.13%)**	**4 (12.50%)**	**3 (9.38%)**	**4 (12.50%)**	**5 (15.63%)**	**3 (9.38%)**
Xavantina	Well	1 (0.95%)	1 (0.95%)	0	1 (3.13%)	1 (3.13%)	0	1 (3.13%)	1 (3.13%)	1 (3.13%)
	Pond	2 (1.90%)	0	0	0	0	0	0	0	0
	Drinkers	0	0	0	0	0	0	0	0	0
	Font	10 (9.52%)	4 (3.81%)	0	0	1 (3.13%)	3 (9.38%)	0	1 (3.13%)	1 (3.13%)
	River	1 (0.95%)	0	0	0	0	0	0	0	0
	Artesian well	6 (5.71%)	3 (2.86%)	0	1 (3.13%)	1 (3.13%)	1 (3.13%)	1 (3.13%)	0	0
	Caxambu font	1 (0.95%)	1 (0.95%)	1 (3.13%)	0	0	1 (3.13%)	0	0	0
	Cistern	0	0	0	0	0	0	0	0	0
Total		**21 (20.00%)**	**9 (8.57%)**	**1 (3.13%)**	**2 (6.25%)**	**3 (9.38%)**	**5 (15.63%)**	**2 (6.25%)**	**2 (6.25%)**	**2 (6.25%)**
Other cities	Well	10 (9.52%)	1 (0.95%)	0	0	0	0	0	0	0
	Pond	5 (4.76%)	2 (1.90%)	0	0	0	1 (3.13%)	0	0	0
	Drinkers	5 (4.76%)	2 (1.90%)	1 (3.13%)	0	0	1 (3.13%)	0	1 (3.13%)	1 (3.13%)
	Font	22 (20.95%)	5 (4.76%)	1 (3.13%)	1 (3.13%)	0	2 (6.25%)	0	1 (3.13%)	1 (3.13%)
	River	6 (5.71%)	1 (0.95%)	0	0	0	1 (3.13%)	0	1 (3.13%)	0
	Artesian well	2 (1.90%)	1 (0.95%)	0	0	0	0	0	0	0
	Caxambu font	0	0	0	0	0	0	0	0	0
	Cistern	0	0	0	0	0	0	0	0	0
Total		**49 (46.67%)**	**12 (11.41%)**	**2 (6.25%)**	**1 (3.13%)**	**0**	**5 (15.63%)**	**0**	**3 (9.38%)**	**2 (6.25%)**
Grand total		**105 (100%)**	**32 (30.46%)**	**3 (9.38%)**	**4 (12.50%)**	**7 (21.88%)**	**13 (40.63%)**	**6 (18.75%)**	**10 (31.25%)**	**7 (21.88%)**

* MDR - Multiple Drug Resistance

and Mustapha (2013) (95 °C for 10 min, 40 cycles of denaturation by 95 °C for 15 s, annealing and extension 60 °C for 45 s). The negative control of the amplification reactions consisted of a sample containing all reagents without DNA.

2.6. Electrophoresis

To visualize PCR amplification products, the gel electrophoresis (1%) technique stained with Ludwig®-brand ethidium bromide, and a molecular marker of 100 bp at 2 kbp of ProteoLadder® brand at 110 V, 150 mA, 110 W for 60 min in the Loccus LPS 300 HC source was used. Then, the gel was read in a photodocumentador with UV light, Loccus brand, with L-PIXEX photodocumentation system.

3. Results

3.1. Isolation of E. coli and the antimicrobial susceptibility test

Tables 3, 4, and 5 show the percentage of *E. coli* isolation, as well as the profile for antimicrobial resistance. Out of 306 samples studied, 44.12% (135/306) were positives for *E. coli* and 66.02% (68/103) were from swine fecal samples, 30.48% (32/105) from water and 35.71% (35/98) from soil. Overall, bacterial resistance was 82.96% (112/135) according to the disk diffusion test, that is, the samples were resistant for at least one of the studied antimicrobial. The highest overall percentages of resistance was for fecal samples and to sulfamethoxazole associated with trimethoprim (63.70% and 75%, respectively), colistin (45.19%

Table 5
Description of soil samples collected for *E. coli* isolation from swine farms of Southern Brazil and their antimicrobial resistance profile.

Cities	Source samples	N° Samples	N° Positives	Beta-lactams		Aminoglycosides	Sulfonamides	Fluoroquinolones	Polymixins	MDR*
				AMC	CTF	GEM	SUT	ENO	COL	
Seara	Pasture	7 (7.14%)	3 (3.06%)	1 (2.86%)	0	0	3 (8.57%)	1 (2.86%)	3 (8.57%)	3 (8.57%)
	Vegetable garden	1 (1.02%)	0	0	0	0	0	0	0	0
	Agriculture	11 (11.22%)	4 (4.08%)	1 (2.86%)	1 (2.86%)	4 (11.43%)	3 (8.57%)	3 (8.57%)	4 (11.43%)	3(8.57%)
	Hillside	10 (10.20)	5 (5.10%)	2 (5.71%)	1 (2.86%)	1 (2.86%)	3 (8.57%)	1 (2.86%)	3 (8.57%)	2 (5.71%)
	Near the Facilities	2 (2.04%)	0	0	0	0	0	0	0	0
Total		**31 (31.63%)**	**12 (12.24%)**	**4 (11.43%)**	**2 (5.71%)**	**5 (14.29%)**	**9 (25.71%)**	**5 (14.29%)**	**10 (28.57%)**	**8 (22.85%)**
Xavantina	Pasture	0	0	0	0	0	0	0	0	0
	Vegetable garden	1 (1.02%)	1 (1.02%)	0	0	1 (2.86%)	1 (2.86%)	0	0	0
	Agriculture	15 (15.31%)	7 (7.14%)	0	0	0	4 (11.43%)	2 (5.71%)	2 (5.71%)	1 (2.86%)
	Hillside	2 (1.02%)	0	0	0	0	0	0	0	0
	Near the Facilities	0	0	0	0	0	0	0	0	0
Total		**18 (18.37%)**	**8 (8.16%)**	**0**	**0**	**1 (2.86%)**	**5 (14.29%)**	**2 (5.71%)**	**2 (5.71%)**	**1 (2.86%)**
Other cities	Pasture	8 (8.16%)	4 (4.08%)	1 (2.86%)	0	1 (2.86%)	2 (5.71%)	0	0	0
	Vegetable garden	3 (3.06%)	0	0	0	0	0	0	0	0
	Agriculture	15 (15.31%)	3 (3.06%)	0	1 (2.86%)	0	2 (5.71%)	0	2 (5.71%)	1 (2.86%)
	Hillside	17 (17.35%)	4 (4.08%)	1 (2.86%)	2 (5.71%)	2 (5.71%)	2 (5.71%)	1 (2.86%)	4 (11.43%)	2 (5.71%)
	Near the Facilities	6 (6.12%)	4 (4.08%)	1 (2.86%)	1(2.86%)	1 (2.86%)	2 (5.71%)	2 (5.71%)	3 (8.57%)	1(2.86%)
Total		**49 (50.00%)**	**15 (15.31%)**	**3 (8.57%)**	**4 (11.43%)**	**4 (11.43%)**	**8 (22.86%)**	**3 (8.57%)**	**9 (25.71%)**	**4 (11.43%)**
Grand total		**98 (100%)**	**35 (35.71%)**	**7 (20%)**	**6 (17.14%)**	**10 (28.57%)**	**22 (62.86%)**	**10 (28.57%)**	**21 (60%)**	**12 (34.29%)**

* MDR - Multiple Drug Resistance

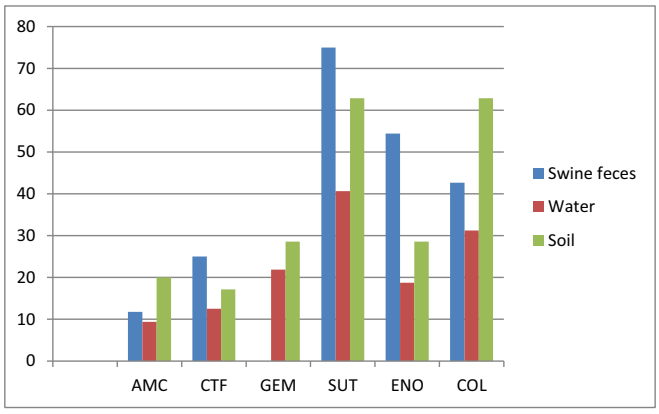

Fig. 1. Profile of antimicrobial resistance of *E. coli* isolated from different sources (swine feces, water and soil) from swine farms of Southern Brazil. Beta-lactams (amoxicillin associated with clavulanic acid - AMC) and third generation cephalosporin (ceftiofur - CTF); Fluoroquinolones (enrofloxacin - ENO); Aminoglycosides (gentamicin - GEM); Sulfonamides (trimethoprim associated to sulfamethoxazole - SUT) and polymyxins (colistin - COL).

and 42.65%, respectively) and enrofloxacin (39.26% and 54.41%, respectively) (Fig. 1). For water and soil, the highest resistance indices were for sulfamethoxazole associated with trimethoprim (40.63% and 62.86%, respectively) and colistin (31.25% and 60%, respectively), with increased resistance to beta-lactams (21.88% and 37.14%, respectively).

Regarding the levels of multidrug resistance (MDR), it was possible to observe that 37.04% (50/135) of the isolates were resistant to at least three different classes of antimicrobials, where strains from feces represented 62% (31/50) of all multiresistant isolates (Table 6).

3.2. Multiple Drug Resistance (MDR) profiles and Multiple Antimicrobial Resistance Index (IRMA)

Table 6 shows the most prevalent multidrug resistance (MDR) profiles and the Multiple Antimicrobial Resistance Index (IRMA) obtained through the disk-diffusion test of *E. coli* strains. The main multirresistance profiles observed were A (GEM-SUT-ENO-COL) with 16% (8/50) of the multiresistant samples, followed by B [CTF-GEM-SUT-ENO-COL] and C [SUT-ENO-COL] with 12% (6/50). Regarding the IRMA results, 78% (39/50) of the multirresistant strains showed values above 0.2, indicating a high potential risk of transmission to humans. Individually, the B (six isolates) and I (two isolates) profiles showed IRMA values of 1, that is, the isolates were resistant to all classes of antimicrobials tested.

3.3. Disk-approximation test for ESBLs

Out of 135 isolates submitted to the disk-approximation test, it was possible to visualize the "phantom zone" in 7.41% (10/135).

3.4. Polymerase Chain Reaction (PCR) to investigate genes related to ESBLs and resistance to fluoroquinolones

The gene *bla*CMY-M2 was detected in 50% (5/10) of the ESBLs-producers, followed by the *bla*TEM-1 genes in 40% (4/10) and *Amp*C with 20% (2/10) (Table 7). However, the genes *bla*SHV-1, *bla*CTX-M2, *bla*OXA-1 and *bla*PSE-1 were not detected. For the genes that confer resistance to the fluoroquinolones, PCR results showed a prevalence of 70% (7/10) for the *qnr*S gene. The *qnr*A and *qnr*B genes were not detected in the *E. coli* strains ESBL-producers.

Table 8 shows the PCR results for the enrofloxacin-resistant strains (n = 46 since 53 isolates were resistant to enrofloxacin and seven were ESBL-producers, which were previously analyzed), where 13.04% of the strains had the presence of *qnr*S gene (6/46). The *qnr*B and *qnr*A genes were not detected.

4. Discussion

Approximately one third of the water samples were contaminated with fecal material. da Malheiros et al. (2009) evaluated the bacteriological contamination of groundwater in the Western region of Santa Catarina and found that 75.94% of them were unfit for human consumption, demonstrating a high microbiological load in the water sources in this region. Several factors can contribute to the contamination of water in rural properties, such as the inefficient management of human and animal waste, as well as the lack of hygiene or protection against rodents and wild animals (Satake et al., 2012). The presence of *E. coli* in fecal samples of swine can easily be explained by the fact that this bacterium is part of the intestinal microbiota, responsible for maintaining the intestinal balance, with the exception of some pathogenic strains that are classified according to some traits of virulence (Romer et al., 2012). Some studies demonstrated the ability of this bacterium to survive and grow for long periods of time in the environment (Bradford et al., 2013).

Another important point to note is the percentage of *E. coli* multiresistant since a third of the isolates showed resistance to at least three classes of antimicrobials and 78% of the multiresistant strains showed IRMA values above 0.2, representing a high risk of transmission to humans (Krumperman, 1983). Resistance to antimicrobial sulfamethoxazole associated with trimethoprim has been widely reported in cases of diarrhea in piglets caused by *E. coli* and also in commensal *E. coli* isolated from swine and the environment (Costa, 2006). The antimicrobial colistin has been widely used as a growth promoter, however recent detections of *mcr*-1 genes conferring bacterial resistance to this

Table 6
Main profiles of Multiple Drug Resistance (MDR) and Multiple Antimicrobial Resistance Index[a] (IRMA) of *E. coli* isolates from swine feces, water and soil.

Identificationprofile	Profile of MDR	Isolated fountain	IRMA	Total	%
A	GEM-SUT-ENO-COL	Feces (4), soil (3), water (1)	0.80	8/50	16
B	CTF-GEM-SUT-ENO-COL	Feces (3), soil (2), water (1)	1	6/50	12
C	SUT-ENO-COL	Feces (4), soil (2)	0.60	6/50	12
D	CTF-GEM-SUT-COL	Feces (3), soil (1)	0.80	4/50	8
E	AMC-SUT-COL	Feces (1), soil (2), water (1)	0.60	4/50	8
F	CTF-SUT-ENO	Feces (4)	0.60	4/50	8
G	GEM-SUT-COL	Feces (1), soil (1), water (1)	0.60	3/50	6
H	GEM-SUT-ENO	Feces (2)	0.60	2/50	4
I	AMC-CTF-GEM-SUT-ENO-COL	Feces (1), soil (1)	1	2/50	4
J,K,L,M,N,O,P,Q,R,S,T	Others[b]	Feces (8), soil (1), water (2)	0.17	1/50	2

% - percentage of samples; Beta-lactams (amoxicillin associated with clavulanic acid - AMC) and third generation cephalosporin (ceftiofur - CTF); Fluoroquinolones (enrofloxacin - ENO); Aminoglycosides (gentamicin - GEM); Sulfonamides (trimethoprim associated to sulfamethoxazole - SUT) and polymyxins (colistin - COL).

[a] Strains with IRMA values above 0.2 are considered to be at high risk of resistance transmission (Krumperman, 1983).

[b] MDR profile that correspond to only 1 (one) isolated.

Table 7
Multiple Antimicrobial Resistance Index (IRMA) and detection of *bla*TEM-1, *bla*CMY-M2, *Amp*C and *qnr*S genes by PCR in *E. coli* ESBL-producer isolated from swine farms of Southern Brazil.

Isolate ID	Source	Collection site	City	IRMA	*bla*TEM-1	*bla*CMY-M2	*Amp*C	*qnrS*
142	Feces	Maternity	Seara	0.6	X	X	X	X
148	Feces	Termination	Seara	0.2		X		X
152	Feces	Maternity	Seara	0.8				X
162	Feces	Gestation	Seara	0.4	X			X
165	Feces	Nursery	Seara	1				X
168	Feces	Gestation	Seara	0.8	X	X		X
184	Feces	Gestation	Seara	0.4		X	X	X
192	Feces	Maternity	Seara	1	X	X		

antibiotic mediated by plasmids lead the Brazilian government to ban its use (Brazil, 2016).

Xiong et al. (2015) reported high concentrations of enrofloxacin, ciprofloxacin and norfloxacin in environments fertilized with animal waste, which could explain the significant antimicrobial resistance rates found in our study. In enterobacteria, one of the major mechanism of resistance to quinolones and fluoroquinolones is mediated by plasmids that carry genes responsible for the synthesis of proteins that in turn block topoisomerases (Hu et al., 2017). These same plasmids may contain other resistance genes, such as those conferring resistance to beta-lactams and cephalosporins, through the production of ESBLs (Jacoby et al., 2014). In this context, more than 400 ESBLs have been described so far and are usually derived from point mutations of the TEM, SHV and CTX-M groups, with 183, 134 and 103 variants, respectively (Barguigua et al., 2011). Abraham (2015) evaluated the resistance of 114 *E. coli* isolated from pigs and detected 14.91% resistance to amoxicillin associated with clavulanic acid and 2.63% to ceftiofur, which were lower than those found in this study (25% and 11.76% for ceftiofur and amoxicillin associated with clavulanic acid, respectively). These data suggest that selective pressure in animal production may explain these findings, especially in regions with high rates of pig breeding (Gebreyes et al., 2017). Significant resistance indices to beta-lactam were detected in *E. coli* strains isolated from water and soil samples. In a study by Jiang et al. (2011), in which the highest prevalence and distribution of multirresistant *E. coli* isolates in China were reported, 27.3% of the water isolates and 18.8% from soil showed resistance to amoxicillin, demonstrating the widespread of resistant bacteria (Egervarn et al., 2017). The lack of Brazilian data related to this matter indicates the need for further studies for better evaluation of the environmental contamination by resistant microorganisms in order to enhance surveillance and to establish preventive measures.

The most prevalent ESBL gene described in previous studies from several sources was CTX-M and its variants CTX-M-2, CTX-M-8 and CTX-M-9 (da Silva and Lincopan, 2012). However, in our study the *bla*CMY-M2 gene was the most prevalent, being positive for 50% of the phenotypically ESBL-producing isolates, followed by the *bla*TEM-1 gene, demonstrating the emergence of other ESBLs encoding genes, increasing concerns about the emergence and spread of these genetic mobile elements. The usual use of third and fourth generation cephalosporins as therapy or food additives in pig production may select high bacterial resistance (Gao et al., 2015), and a matter of growing concern is that genes encoding ESBLs are frequently found (Xu et al., 2015), a fact related to the results obtained in our study, where out of ten *E. coli* ESBLS-producers, seven showed the *qnr*S gene, and 142 isolates showed, concomitantly, the genes *bla*TEM, *bla*CMY-M2, *Amp*C and *qnr*S. The identification of ESBL genes encoding ESBLs in *E. coli* isolates of environmental origin enhances the need for adequate rural sanitation, considering the possibility of contamination of water and food with resistant microorganisms. Brazil and the Western region of Santa Catarina are important producers of pork meat, and the possibility to disseminate these genes to other pathogenic microorganisms through mobile genetic elements such as plasmids, integrons and transposons by bacterial conjugation can considerably affect animal production, as well as human health.

Table 8
Results of Multiple Antimicrobial Resistance Index (IRMA) and detection of *qnr*S gene by PCR in enrofloxacin resistant *E. coli* isolated from swine farms of Southern Brazil.

Isolated	Source	Collection point	City	IRMA	*qnrS*
177	Feces	Maternity	Seara	0.8	X
179	Feces	Maternity	Seara	1	X
189	Feces	Maternity	Seara	0.6	X
232	Feces	Nursery	Xavantina	1	X
273	Feces	Maternity	Xavantina	0.4	X
277	Feces	Termination	Xavantina	0.4	X

5. Conclusion

It is possible to conclude that most of the strains of *E. coli* isolated were resistant to antimicrobials and carry genes of resistance to quinolones, fluoroquinolones and ESBLs and because of that they are resistant to these antimicrobials. Considering that Brazil is a large meat producer with unknown status regarding the antimicrobial resistance our findings are important for public health. In addition, the dissemination of resistance genes between strains of the same species or even between different species may cause serious diseases in humans and animals with difficult treatment, highlighting the need of rational use of antimicrobials and adequate animal waste treatment.

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References

ABPA. Brazilian Association of Animal Protein, 2017. Summary of the pig sector. Available at:. http://abpa-br.com.br/setores/suinocultura/resumo, Accessed date: 23 October 2017.

Abraham, S., 2015. First detection of extended-spectrum cephalosporin- and fluoroquinolone-resistant *Escherichia coli* in Australian food-producing animals. J. Glob. Antimicrob. Resist. 3, 273–277.

Alcaine, S.D., Sukhnanand, S.S., Warnick, L.D., Su, W.L., Mcgann, P., Mcdonough, P., Wiedmann, M., 2010. Ceftiofur-resistant *Salmonella* strains isolated from dairy farms represent multiple widely distributed subtypes that evolved by independent horizontal gene transfer. Antimicrob. Agents Chemother. 49 (10).

ANVISA - National Agency of Sanitary Surveillance, 2003. Standardization of the Sensitivity Tests to Antimicrobial by Disc-diffusion: Approved Standard. eighth edition. (Brazil).

Barguigua, A., El Otmani, F., Talmi, M., Bourjilat, F., Haouzane, F., Zerouali, K., et al., 2011. Characterization of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from the community in Morocco. J. Med. Microbiol. 60, 1344–1352.

Bradford, S.A., Morales, V.L., Zhang, W., Harvey, R.W., Packman, A.I., Mohanran, A., Welty, C., 2013. Transport and fate of microbial pathogens in agricultural settings. Crit. Rev. Environ. Sci. Technol. 43, 775–893.

Brazil, 2016. Normative Instruction N 45, Dated November 22, 2016 (Official Journal of the Union).

Caldorin, M., Almeida, I.A.Z., Peresi, J.T.M., Alves, E.C., 2013. Occurrence of shiga toxin-producing *Escherichia coli* (STEC) in Brazil and its importance in public health. Boletim Epidemiológico Paulista. vol. 10, pp. 4–20.

Chen, S., Zhao, S., White, D.G., Schroeder, C.M., Lu, R., Yang, H., Mcdermott, P.F., Ayers, S., Meng, J., 2004. Characterization of meats *Salmonella* serovars isolated from retail multiple-antimicrobial-resistant. Appl. Environ. Microbiol. 1, 1–7.

Clinical and Laboratory Standarts Institute – CLSI, 2018. Performance standarts for antimicrobial susceptibility testing. Twenty-fifth Informational Supplement.

Costa, M.M.D., 2006. Epidemiological, molecular and antimicrobial resistance profile of *Escherichia coli* isolated from pig farms in Southern Brazil. Pesqui. Vet. Bras. 26, 5–8.

Egervarn, M., Englund, S., Ljunge, M., Wiberg, C., Finn, M., Lindblad, M., Borjesson, S., 2017. Unexpected common occurence of transferable extended spectrum cephalosporinase producing *Escherichia coli* in Swedish surface waters used for drinking water supply. Sci. Total Environ. 587–588, 466–472.

EPAGRI - Company of Agricultural Research and Rural Extension of Santa Catarina State, 2017. Production of beef, pork and chickens slaughtered, Brazil and Santa Catarina from 2007 to 2016. Available at:. http://www.epagri.sc.gov.br/?page_id=2623, Accessed date: 23 February 2018.

Frye, J.G., Cray, P.J., 2007. Prevalence, distribution and characterization of ceftiofur resistance in *Salmonella* enterica isolated from animals in the USA from 1999 to 2003. Int. J. Antimicrob. Agents 30, 134–142.

Gao, L., Tan, Y., Zhang, X., Hu, J., Miao, Z., Wei, L., Chai, T., 2015. Emissions of *Escherichia coli* carring extended-spectrum beta-lactamase resistance from pig farms to the surrounding environmental. Int. J. Environ. Res. Public Health 12, 4203–4213.

Gebreyes, W.A., Wittum, T., Habing, G., Alali, W., Usui, M., Suzuki, S., 2017. Chapter 4: spread of antibiotic resistance in food animal production systems. Foodborne Diseases, 3rd ed., pp. 105–130.

Hu, Y.S., Shin, S., Park, Y.H., Park, K.T., 2017. Prevalence and mechanism of fluoroquinolone resistance in *Escherichia coli* isolated from swine feces in Korea. J. Food Prot. 80, 1145–1151.

Jacoby, G.A., Strahilevitz, J., Hooper, D.C., 2014. Plasmid-mediated quinolone resistance. Microbiol. Spectr. 2, 1–24.

Jarlier, V., Nicolas, M.H., Fournier, G., Philippon, A., 1988. Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in *Enterobacteriaceae:* hospital prevalence and susceptibility patterns. Rev. Infect. Dis. 10, 867–878.

Jiang, H.X., Lu, D.H., Chen, Z.L., Wang, X.M., Chen, J.R., Liu, Y.H., Liao, X.P., Liu, J.H., Zeng, Z.L., 2011. High prevalence and widespread distribuition of multi-resistant *Escherichia coli* isolates in pigs and poultry in China. Vet. J. 187, 99–103.

Krumperman, P.H., 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Appl. Environ. Microbiol. 46, 165–170.

da Malheiros, P.S., Schäfer, D.F., Herbert, I.M., Capuani, S.M., Da Silva, E.M., Sardiglia, C.U., Scapin, D., Rossi, E.M., Brandelli, A., 2009. Bacteriological Contamination of Groundwater of the Western Area of Santa Catarina, Brazil. 68. Revista Instituto Adolf Lutz, pp. 305–308.

Moumoni, A., Diagbouga, S., Nadembéga, C., Dabiré, A.M., Salah, F., Obiri-yeboah, D., Soubéiga, S.T., Ouattara, A.K., Zohoncon, T., Djigma, F., Langendorf, C., Simporé, J., 2017. Quinolone resistance (*qnr*) genes in fecal carriage of extended spectrum beta-lactamases producing enterobacteria isolated from children in Niger. Curr. Res. Microbiol. Biotechnol. 5, 953–957.

Quinn, P.J., Markey, B.K., Carter, M.E., Donnelly, W.J.C., 2005. In: Weiss, L.H.N., Weiss, R.D.M. (Eds.), Infectious Veterinary Microbiology and Disease. Porto Alegre, Artmed, p. 12.

Rochelle-Newall, E., Nguyen, T.M., Sengtaheuanghoung, O., Ribolzi, O., 2015. A short review of fecal indicator bacteria in tropical aquatic ecosystems: knowledge gaps and future directions. Front. Microbiol. 6, 308.

Romer, A., Wieler, L.H., Schierack, P., 2012. Analyses of intestinal commensal *Escherichia coli* strains from wild boars suggest adaptation to conventional pig production conditions. Vet. Microbiol. 161, 122–129.

Satake, F.M., Assunção, A.W.A., Lopes, L.L.G., Amaral, L.A., 2012. Water Quality in Rural Properties Located in the Hydrographic Basin of the Rich Stream, Jaboticabal, SP. ARS Veterinária, Jaboticabal, SP. vol. 28, 1 pp. 48–55.

da Silva, K.C., Lincopan, N., 2012. Epidemiology of extended-spectrum beta-lactamases in Brazil: clinical impact and implications for agribusiness. J. Bras. Patol. Med. Lab. 48, 91–99.

Silva, C., Oliveira, A., Bezerra, P., Evangelista, J., 2015. *Escherichia coli* in swine. Clinical aspects. A review. Rev. Bras. Hig. Sanid. Anim. 9, 123–131.

Singh, P., Mustapha, A., 2013. Multiplex TaqMan detection of pathogenic and multi-drug resistant *Salmonella*. Int. J. Food Microbiol. 2, 213–218.

World Health Organization, 2014. Antimicrobial Resistance: Global Report os Surveillance 2014. WHO, Geneva, Switzerland.

Xiong, W., Sun, Y., Ding, X., Zhang, Y., Zhong, X., Liang, W., Zeng, Z., 2015. Responses of plasmid-mediated quinolone resistance genes and bacterial taxa to (fluoro)quinolones-containing manure in arable soil. Chemosphere 119, 473–478.

Xu, G., Na, W., Wang, H., Zhang, X., 2015. Prevalence and characteristics of extended-spectrum Beta-lactamase genes in *Escherichia coli* isolated from piglets with post-weaning diarrhea in Heilongjiang province, China. Front. Microbiol. 6, 1–9.

Yeh, J.C., Lo, D.Y., Chang, S.K., Chou, C.C., Kuo, H.C., 2017. Prevalence of plasmid-mediated quinolone resistance in *Escherichia coli* isolated from diseased animals in Taiwan. J. Vet. Med. Sci. 79, 730–735.

Yue, L., Jiang, H.X., Liao, X.P., Liu, J.H., Li, S.J., Chen, X.Y., Chen, C.X., Lu, D.H., Liu, Y.H., 2008. Prevalence of plasmid-mediated quinolone resistance *qnr* genes in poultry and swine clinical isolates os *Escherichia coli*. Vet. Microbiol. 132, 414–420.