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Salmonella and antimicrobial resistance in an animal-based agriculture river system

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HIGHLIGHTS

- The highest number of samples contaminated with *Salmonella* sp. was associated with Sites with high stocking density.
- 30 different serovars were found and at least 11 per monitoring Site.
- 50.5% were susceptible to 21 antimicrobials and 54 different profiles were found.
- 49.5% of the isolates were resistant to at least one antimicrobial.
- Multi-resistance occurred in 18% of isolates.

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ABSTRACT

The aim of this study was to examine the *Salmonella* serovars and antimicrobial resistance within an animal-based agriculture river system. The study area consisted of a 1345 ha upper part of Pinhal catchment. A total of 384 samples were collected in four years of monitoring. *Salmonella* was isolated from 241 samples (62.7%), resulting in 324 isolates. The highest number of *Salmonella* sp. occurred in samples associated with sites with high stoking density animal unit per hectare. It was possible to demonstrate the variability of serovars in the study area: 30 different serovars were found and at least 11 per monitoring site. Thirty-three potentially related isolates were genotyped by PFGE, one major clone was observed in serovar Typhimurium, which occurred in animal feces (swine and bovine), and different sites and samplings proving the cross-contamination and persistence of this specific clone. Among 180 isolates submitted to an antimicrobial susceptibility test, 50.5% were susceptible to all 21 antimicrobials tested and 54 different profiles were found. In the current study, 49.5% of the tested isolates were resistant to at least one antimicrobial, and multi-resistance occurred in 18% of isolates. Results indicate a close interaction between animal-based agriculture, *Salmonella*, and antimicrobial resistance.

1. Introduction

The demand for animal protein in the next years is anticipated to increase significantly. Economists estimate that by the year 2050, global meat production must increase by 73% to meet the expected 43% boost to the world's population. Three other basic factors driving global demand for animal protein are economic growth and income, the rising middle-class of countries, and urbanization. Broken down by species, to meet anticipated animal protein demand, global poultry production will need to increase by 125%, followed by sheep and goat meat, 78%; beef, 58%; and pork, 37% (Barr, 2012).

The growing consumption of animal products in developing countries demands a proportional increase in animal production. Considering the social inclusion of people in developing countries as consumers, the position of these countries as livestock producers, and that the majority of production must occur in a conventional manner using large amounts of antibiotics, studies linking animal production, use of antibiotics, and environmental quality should be done.

Emergence of antimicrobial-resistant human and animal pathogens is regarded as a fateful complication of antimicrobial abuse in livestock. The World Health Organization recently called for the elimination of antibiotics for growth promotion in agriculture that is also used in human medicine. The European Union (EU) initiated several actions including

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the removal of all antimicrobials used as growth-promoting substances in the livestock industry (Regulation EC 1831/2003) (García-Feliz et al., 2008). In order to ensure responsible and cautious use of antimicrobials in livestock and veterinary medicine, and to monitor the antimicrobial resistance emergence, the major livestock-producing countries have established their own national surveillance systems, such as NARMS of the United States (Food and Drug Administration, 2010).

Peak et al. (2007) established that much of the work that led to these conclusions focused on resistance transfer through the food-supply—however, resistance might also migrate away from operations via water. Mackie et al. (1998) detected that animal manure may contain antimicrobial compounds, which result from on-farm livestock management. The United States Environmental Protection Agency (2012) concluded that antimicrobials contained in manure and biosolids may enhance selection of resistant bacteria by entering the aquatic environment through pathways of diffuse pollution.

Whether or not antibiotics have a deleterious effect on ecosystem health cannot be addressed by experimental studies only, as laboratory studies often have a limited relevance to the environment (Ingerslev and Halling-Sørensen, 2001). One proposed way to solve this task is the collection of data gained from environmental sampling (Boxall et al., 2003).

Salmonella is a public health concern due to the number of cases per year, and the most common route of infection is through food products derived from animals such as poultry and pigs, but many strains are resistant to several antimicrobial agents (Hur et al., 2012; Duijkeren et al., 2003). In Rio Grande do Sul, the southern state of Brazil, *Salmonella* has been isolated in foodborne outbreaks and strains presenting antimicrobial resistance have been identified (Welker et al., 2010; Geimba et al., 2005; Costalunga and Tondo, 2002).

A high percentage of antibiotic-resistant *Salmonella* strains from swine production have been demonstrated in Brazil. Kich et al. (2011) found that 83% (475/572) of *Salmonella* strains were resistant to at least one antibiotic, and 43% (246/572) were resistant to four or more antibiotics. In a recent summary of resistance trends, the NARMS Executive Report indicated that in 2007, 53.9%, 72% and 43.1% of non-typhoidal *Salmonella* isolates from chickens, cattle, and swine, respectively, were resistant to at least one antimicrobial agent, which is similar to those reported in 1996 (FDA, 2010).

The aim of this study was to examine the *Salmonella* serovars and antimicrobial resistance within an animal-based agriculture river system. It will contribute to our understanding of the relationship between livestock and environment quality, and human and animal health.

2. Material and methods

The study area consisted of a 1345 ha of the upper Pinhal catchment. Pinhal is located in Concordia, Santa Catarina, Brazil. The highest points of nearby hills reach elevations of 724 m, while the lowest parts are at about 574 m above sea-level. Pinhal is a first-order stream and later forms a second-order stream with the Guilherme River and Rancho Grande River that drains into the Uruguay River. Uruguay basin has a fundamental importance because it is a trans-boundary basin; it has an extension of 2200 km. Argentina, Bolivia, Brazil, Paraguay, and Uruguay share the basin. In Brazil, the Uruguay River divides the states of Santa Catarina and Rio Grande do Sul.

The Pinhal catchment is typically agricultural. The area is predominantly agricultural, with dairy cow, broiler, and pig farming and a large proportion of land area devoted to corn, pastures, and forage crops. Detailed descriptions about the number of animal units and stocking density in each monitoring Site can be found in Palhares et al. (2011).

Antimicrobial-use information for individual farms was not collected as part of this study. We know that each feedlot has its own antibiotic strategy, which varies depending upon the purpose, timing, and amount and type of antibiotic used. All of the antimicrobials we evaluated are approved for use in livestock and broiler for therapeutic purposes and/or growth promotion. Fig. 1 shows the inputs and outputs of antibiotic resistance determinants along the river.

The interval between manure application and sample collection was not determined, nor was the amount of manure applied to the crop fields monitored. The animal feeding operations and agricultural fields upon which manure from the operations was applied could be adjacent or separate.

2.1. Sampling

Eight monitoring Sites were selected and monitored monthly for four years (384 samples), from October 2006 to October 2010. The Sites were located at places where conditions were most representative and homogeneous, away from areas with point sources, mixing zones, and non-point sources. The description of each Site is presented in Table 1. Because convenience sampling was employed for this study, the samples were not considered to be representative of any particular livestock-raising practice or manure management strategy.

A total of 180 samples were taken from 14 farms located around the Pinhal catchment in the 4th year of the monitoring. The number of samples per farm ranged from 5 to 14 according to animal agriculture facilities. Feces from dairy cow and pigs and soil in corn and forage crops areas were taken. Feces were collected in the rectum of 10% of the animals confined in each facility. All facilities were sampled. One animal corresponded to a single sample, and the sample was composed by mixing samples from each animal. Soil samples were collected in areas where animal wastes were used as fertilizer.

2.2. Salmonella isolation

At each Site, in-stream samples were collected on the same day and stored in pre-sterilized glass bottles and retained in the dark on ice until being returned to the laboratory for final processing (<24 h since sample collection).

Salmonella was isolated from running water by immersion of a sterile pad in the water for 48 h in all Sites. The pad consists of cotton wool wrapped in a square of surgical gauze and securely tied at one end with a long piece of strong string (Quinn et al., 1994). The pad is retrieved and placed in a jar containing 225 ml of buffered peptone water for 24 h at 37 °C as a pre-enrichment step. The *Salmonella* detection followed the procedures described in ISO 6579 and was adapted from Michael et al. (2003). Each confirmed isolate was serotyped at the National Reference Center, Instituto Osvaldo Cruz, Rio de Janeiro, Brazil using a standard slide agglutination assay.

2.3. PFGE of Salmonella isolates and data analysis

In order to relate *Salmonella* isolates from the farming activities and river contamination, thirty-three potentially related isolates (same

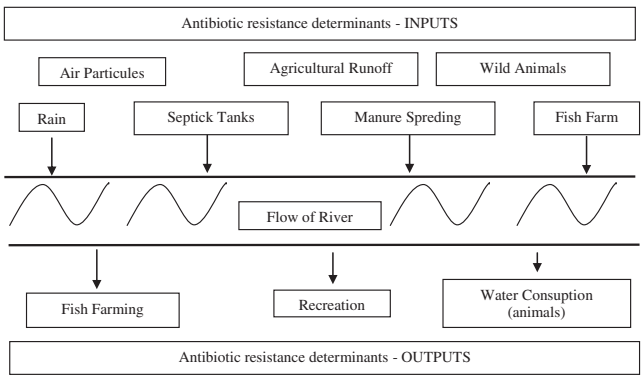

Fig. 1. Inputs and outputs of antibiotic resistance determinants along the Pinhal River.

Table 1
Site description in Pinhal catchment.

Site	Area (ha)	Altitude (m)	Land use
1	23.3	724	One of the principal tributaries of the Pinhal River, lots of spring contribute to this tributary, there isn't riparian buffer, soil with erosion, animal manures go direct to the river
2	67.2	705	Another principal tributary of the Pinhal River, there aren't any economy activity, forest is preserved and in agreement with the environmental Brazilian law
3	209.6	636	Upright of this there are farms with pigs, poultry and dairy, the agricultural area is occupied with corn, pastures, and grasslands, and in these areas organic and inorganic fertilizers are used, the riparian buffer is not totally preserved
4	197.6	630	Mixing zone of tributaries from Sites 1 and 3 and from Site 2
5	411.6	629	Pig farm beside the river and the tank of waste is 7 m from the river, riparian buffer in a strip of 10 m, but only from one side of the river, upright of this Site there is a little village and farms with pigs, poultry and dairy, riparian buffer is reduced in both sides along the river, lands with corn and pastures
6	112.0	619	Upright farms with pigs, poultry and dairy, riparian buffer is reduced in both sides of the river, it receives the effluents of a fish farming integrated with pigs, lands with corn and pastures
7	150.2	607	Downstream from the center of the village, where there is a school, and cemetery, also suffers influences of agricultural activities and livestock, buffer zone more present than in the other Sites
8	173.7	574	Pigs and dairies, farms around, land with a rise slope and with cultivation of corn, natural and cultivated pasture and pines, good condition of the riparian buffer in some margins of the river

serovar occurring in farm's and river's isolates) were submitted to genotyping by pulsed field gel electrophoresis (PFGE). The serovars included in this analysis were driven by the farm isolates: Typhimurium and Panama, most prevalent in swine production, Rubislaw that occurred mostly in bovine samples and Oranienburg isolated from soil.

The bacterial suspension was embedded in agarose, lysed, washed, and digested with the restriction enzyme, XbaI (New England Biolabs, Beverly, MA) overnight (12–16 h) at 37 °C essentially as described in the Centers for Disease Control and Prevention (Atlanta, GA) "One-Day (24–28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* by pulsed field gel electrophoresis (PFGE)" (Clinical and Laboratory Standards Institute, 2004 http://www.cdc.gov/pulsenet/protocols.htm) (Ribot et al., 2006). Electrophoresis was performed in a 1% agarose gel using 0.5X Tris–borate–EDTA buffer on a Chef Mapper XA (BioRad Laboratories, Hercules, CA) at 6 V/cm for 19 h at 14 °C with an initial switch time of 2 min and 16 s and a final switch time of 63.8 s. Gels were stained for 30 min at room temperature with ethidium bromide (Invitrogen, Carlsbad, CA), destained, and photographed. *Salmonella choleraesuis* subspecies Braenderup (ATCC# BAA-664) was included as a reference. Pattern images were acquired using a Kodak Gel Logic 2200 system and analyzed using the BioNumerics software program, Version 2.0 (Applied Maths BVBA, Saint-Martens-Latem-Belgium). Similarities between isolate fingerprints were determined on the basis of the Dice correlation coefficient. A band position tolerance of 1.7% was used for the analysis of PFGE patterns (Carriço et al., 2005). Dendrograms were generated by unweighted pairwise grouping with mathematical averaging (UPGMA). Isolates were considered as having the same pulsotype when the number and location of the bands were indistinguishable. Isolates with one band difference were considered to be of distinct pulsotypes.

2.4. Antimicrobial susceptibility-resistance profiling

A subset of 180 *Salmonella* isolates was profiled according to the isolates' susceptibility-resistance to 21 antimicrobials using the antimicrobial susceptibility test (AST). Disk diffusion AST was carried out as described by the Clinical and Laboratory Standards Institute (CLSI) (2005).

The following antimicrobial agents and each concentration were analyzed: nalidixic acid (NAL), 30 μg; amoxicillin/clavulanic acid (AMC), 20/10 μg; amikacin (AMI), 30 μg; ampicillin (AMP), 10 μg; kanamycin (K), 30 μg; cephalothin (CF), 30 μg; ceftazidime (CAZ), 30 μg; ceftiofur (CEF), 30 μg; ciprofloxacin (CIP), 5 μg; chloramphenicol (CHL), 30 μg; colistin (CL), 10 μg; doxycycline (DX), 30 μg; enrofloxacin (ENR), 5 μg; streptomycin (STR), 10 μg; florfenicol (FLO), 30 μg; gentamicin (GEN), 10 μg; neomycin (NEO), 30 μg; norfloxacin (NOR), 10 μg; sulfametazol + trimethoprim (SXT), 1.25/23.75 μg; tetracycline (TET), 30 μg; and trimethoprim (TMP), 5 μg.

Disk diffusion AST was carried out as described by the Clinical and Laboratory Standards Institute (2005). Each organism was first classified as resistant, intermediate-resistant, or susceptible to the agent. *E. coli* ATCC 25922 was used as a control strain of known antibiotic susceptibility (CLSI, 2008).

3. Results

A total of 384 samples were collected in 48 days, monthly distributed, in four years of monitoring. *Salmonella* was isolated in 62.7% (241/384) of samples resulting in 324 isolates. Among the Sites, *Salmonella* ranged from 9.54% positive (23/241) at Site 1 to 14.94% positive (36/241) at Site 5. Fig. 2 shows the number of *Salmonella* positive samples by each Site.

The prevalence increased downstream. The highest number of *Salmonella* sp. isolates by Site occurred in samples associated with highest animal stocking density, Sites 5, 75% (36/48) and 6, 73% (35/48). Site 1 had the lowest frequency of *Salmonella* positive samples, 48% (23/48).

When examined over the four year study period, Year 4 had the lowest counts of positive samples 18% (44/241) and Year 3 the highest, 28% (67/241) (Fig. 3). *Salmonella* prevalence ranged from detectable in one Site, February and August 2010, to all Sites. Seasonally, detection frequency was greatest in the summer and lowest in the winter.

The identical serovar from the same sample was considered a duplicate and counted once. It results in 227 *Salmonella enterica* subspecies *enterica* being completely serotyped. The occurrence is demonstrated in Fig. 4. The serovars Panama, Infantis, Typhimurium and Derby of subspecies *enterica* occurred with the highest frequency. The subspecies Diarizonae and Houtenae were found once each. In Brazil, *Salmonella* Typhimurium and Panama are the most prevalent serotypes isolated from animals, especially swine (Pereira, 2007; Kich et al., 2007, 2011).

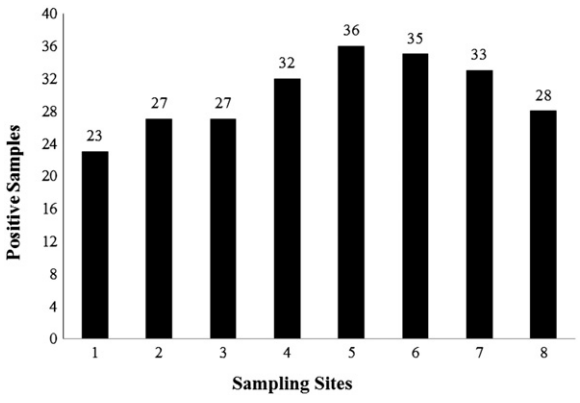

Fig. 2. *Salmonella* positive samples (N = 241) by each Site during the monitoring period.

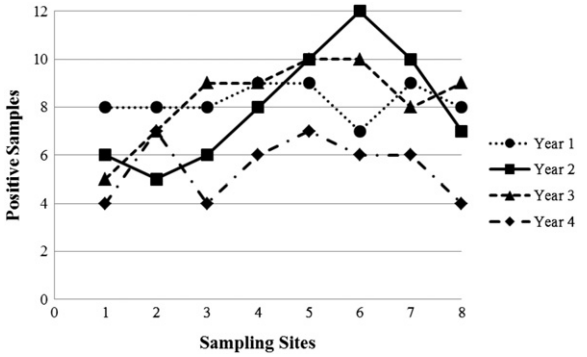

Fig. 3. Distribution of *Salmonella* positive samples during the years. Year 1, 27% (66/241); Year 2, 27% (64/241); Year 3, 28% (67/241); Year 4, 18% (44/241).

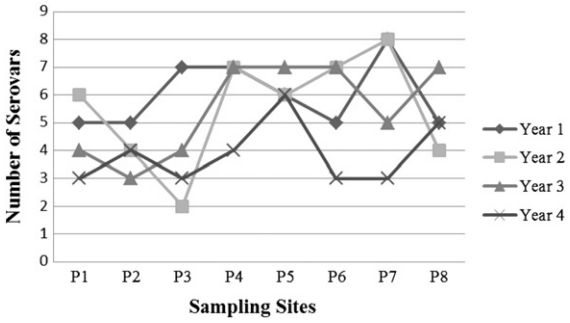

Fig. 5. Number of different *Salmonella* serovars found by sampling Site and year of monitoring.

From all isolates, 30 different serovars were distributed over the sampled area. This fact demonstrated the high variability of this organism in the catchment. Three serovars occurred in more than 50% of samples, Panama (47/48), Infantis (33/48), and Typhimurium (24/48). At least, 8 serovars per Site per year were found (Fig. 5), suggesting that a wide range of *Salmonella* serovars play a role in river microbiological water quality.

In order to investigate the contamination source, 14 farms were visited in the last year of this study. Six serovars were identified, Typhimurium, Derby, Panama, Rubislaw, Oranienburg, and Livingstone, from 33/180 positive samples. The comparison, by clustering analysis, among farms and river samples from four serovars representative of the potential contamination source, feces from swine and bovine and soil, is depicted in Fig. 6.

It was possible to compare isolates from four serovars: Typhimurium (23), Panama (4), Rubislaw (4), and Oranienburg (2). One major clone group was observed in serovar Typhimurium, which occurred in swine and bovine feces at different Sites and sampling times as well (Fig. 6.a). Three minor clone groups were found at different Sites, two in Typhimurium (Fig. 6.a) and one in Panama (Fig. 6.b). Finally, we observed one clone of serovar Rubislaw isolated from swine and bovine feces in three different samplings.

Antimicrobial susceptibility/resistance distribution of 180 *Salmonella* isolates is demonstrated in Table 2. More than 50% (91/180) were susceptible to all 21 antimicrobials tested and 54 different profiles were found (Table 3). In the current study, 49.5% of the isolates were resistant to at least one antimicrobial and multi-resistance occurred in 18% of the isolates. Multi-resistance by sampling Site is demonstrated in Fig. 7.

More than 10% of isolates were resistant to ampicillin, doxycycline, nalidixic acid, neomycin, kanamycin, and tetracycline, with the last two presenting the highest resistance, 21.1% and 13.8%, respectively.

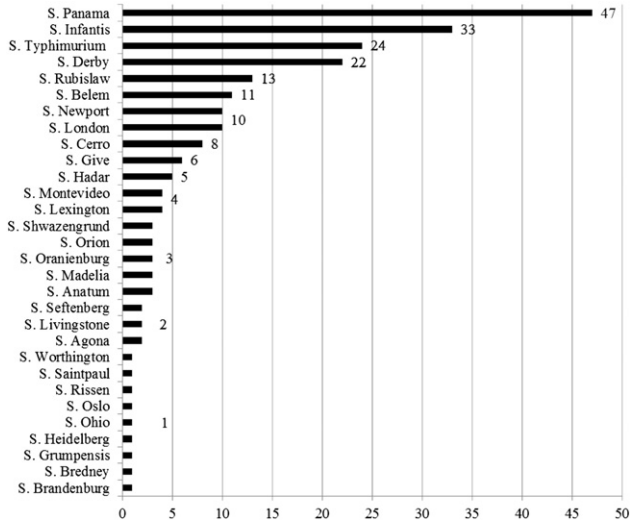

Fig. 4. Occurrence of *Salmonella enterica* subspecies *enterica* serovars by sample.

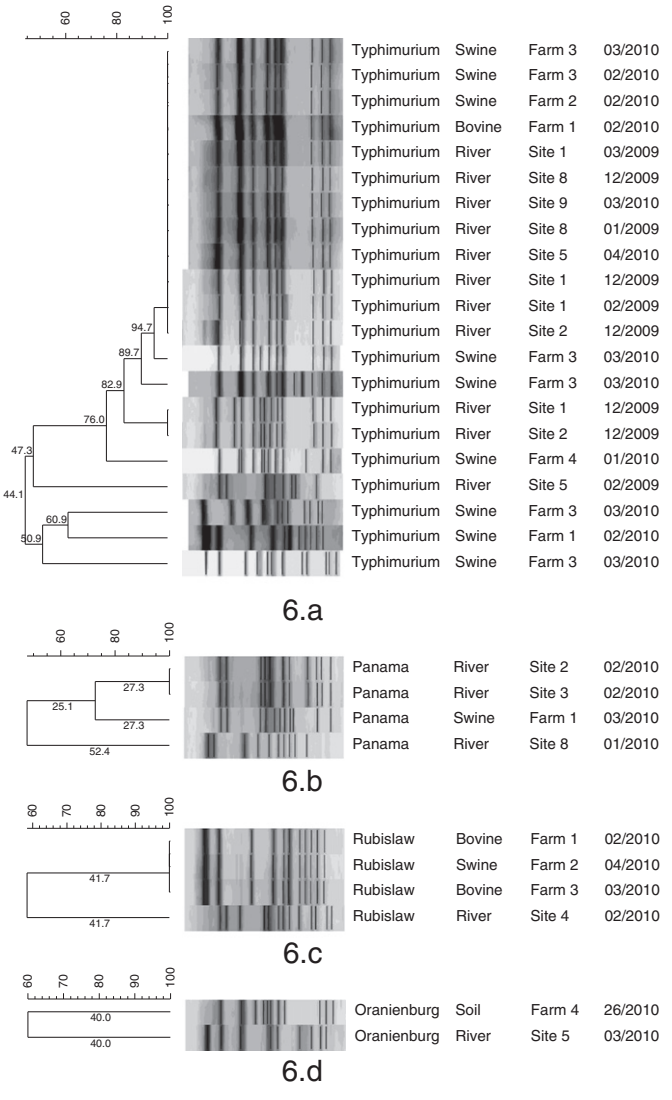

Fig. 6. XbaI pulsed field gel electrophoresis (PFGE) patterns of the *Salmonella* isolates from water and farms located in the Pinhal River.

Table 2
Distribution of susceptibility/resistance of 180 *Salmonella* isolates.

Antimicrobial	Susceptibility		Intermediate		Resistance	
	N	%	N	%	N	%
Amikacin (AMI)	177	98.33	1	0.56	2	1.11
Amoxicillin/clavulanic acid (AMC)	166	92.22	3	1.67	11	6.11
Ampicillin (AMP)	143	79.44	16	8.89	21	11.67
Ceftazidime (CAZ)	174	96.67	1	0.56	6	3.33
Ceftiofur (CEF)	176	97.78	2	1.11	2	1.11
Cephalothin (CF)	169	93.89		0.00	11	6.11
Chloramphenicol (CHL)	172	95.56	2	1.11	6	3.33
Ciprofloxacin (CIP)	174	96.67	5	2.78	1	0.56
Colistin (CL)	172	95.56	4	2.22	4	2.22
Doxycycline (DX)	152	84.44	4	2.22	24	13.33
Enrofloxacin (ENR)	178	98.89	1	0.56	1	0.56
Florfenicol (FLO)	176	97.78	1	0.56	3	1.67
Gentamicin (GEN)	171	95.00		0.00	9	5.00
Kanamycin (K)	124	68.89	18	10.00	38	21.11
Nalidixic acid (NAL)	153	85.00	7	3.89	20	11.11
Neomycin (NEO)	16	8.89	142	78.89	22	12.22
Norfloxacin (NOR)	173	96.11	2	1.11	5	2.78
Streptomycin (STR)	176	97.78	2	1.11	4	2.22
Sulfametazol + trimethoprim (SXT)	171	95.00	5	2.78	7	3.89
Tetracycline (TET)	150	83.33		0.00	25	13.89
Trimethoprim (TMP)	167	92.77		0.00	12	6.67

4. Discussion

Salmonella was present at all Sites and throughout the monitoring period (Figs. 2 and 3). In mixed-use watersheds, fecal contamination can be of livestock, human, or wildlife origin. In this study *Salmonella* were frequently detected from a majority of Sites influenced by varying degrees of agriculture and, in particular, livestock and poultry production. Sigua et al. (2009) suggested that a spatial pattern of bacterial water quality is evident, which can be linked to the different land-uses and associated practices (presence or absence of animal activities, and presence or absence of crops).

Salmonella detection among the Site with highest occurrence, Site 5, 75% (36/48), and with the lowest, Site 1, 48% (23/48), was 27%. Site 2, where there was no economy activity, and the forest was preserved in agreement with the Brazilian environmental law, showed an occurrence of 56% (27/48). In Site 1 there is no riparian buffer zone and dairy manures went direct to the river. We propose two hypotheses for it happened in Site 2: presence of wildlife and contact of wildlife with livestock positive to *Salmonella*. Jamieson et al. (2004) concluded that one of the major difficulties in microbial pollution assessment is characterizing wildlife or "background" levels of contamination. Wildlife, such as waterfowl, can be a significant contributor to fecal pollution within rural watersheds.

The highest occurrences in Sites 5 and 6 were related to animal stocking density and that swine and broiler farms were established in an industrial system, when animals have more contact with their feces inducing a fecal–oral cycle contamination. This also enables better environmental conditions for the development of the bacteria.

Site 8 also had a lower occurrence, 58% (28/48). As in Sites 1, 2, and 3, relief on this Site was rougher, providing more choppy waters with higher concentrations of dissolved oxygen (Palhares et al., 2011). Other physical and chemical water quality parameters can also influence the presence of *Salmonella*. Leclerc et al. (2002) showed that the presence of enteric bacteria in the aquatic environment depends on a variety of parameters, which include nutrients. Palhares et al. (2012), monitoring the Pinhal River, observed a relationship among Sites with higher animal stocking densities and high concentrations of nitrate.

Year 4 had the lowest occurrence of *Salmonella* for all Sites 18% (44/241). This may be related to the reduction of environmental pressure by the number of animal units, but this information has not been verified. During the monitoring period, the Environmental State Agency proposed an Environmental Adjustment Contract (EAC) for pig farms. Operations had to improve the waste management and recovered 10 m of riparian zone on both sides of the river. Managements proposed by EAC improved environmental quality through the years. It occurred between Years 2 and 3. The lowest positive samples in Year 4 could be the result of this process. Results of other years did not show a pattern of distribution and reduction of positive samples between Sites.

Salmonella occurrence was highly variable across months. In the Pinhal region autumn and winter are the driest seasons. The rainfall probability is high during spring. Strongest rains occurred in September and October. In Year 1, October, March and April had occurrence to all Sites. In Year 2, it happened in March and August and in Year 3, November and January. Year 4 showed occurrence to all Sites in November and December. During the first two years of monitoring, occurrence peak was verified at the end of summer (March and April) and winter (August). In the last two years of monitoring they were verified in the summer. Vereen et al. (2013) indicated that *Salmonella* infections among humans generally peak in summer months but environmental studies often show varied seasonal peaks for these pathogens, with some suggesting higher prevalence in summer months.

August and September are the months which there are the soil preparation period for corn crops when farmers use a lot of livestock and broiler manure as fertilizer, but only in Year 2 the occurrence peaked in this season. The use of animal waste as fertilizer can be a way to carry *Salmonella* to waters. Controlling manure application rate, analyzing manure nutrients, and considering climate and soil conditions, and plant characteristics are means to reduce the risk of some pathogens moving with runoff. Farmers on the Pinhal catchment did not use the animal wastes considering these parameters, but this uncontrolled use cannot be related with the *Salmonella* occurrence.

According to Venglovsky et al. (2009) it is a well-established fact that bacterial pathogens may persist for long periods in animal manures under typical farm conditions. This may be extended when the temperatures are low, moisture remains optimal, and aeration is not used. It is possible that a combination of extended storage coupled with other processes, including aeration, may be beneficial in reducing pathogen-loading in waste material. Farms only had storage systems without aeration of pig waste. Santa Catarina environment law determines 120 days in stored system. Broiler wastes were composted before agricultural use and dairy was directly disposed of in the soil.

Table 3
Salmonella resistance profile found in the study (54 profiles).

Resistance profile[a]	Number of antimicrobial resistance	Number of isolates	Serovars[b]
Amc	1	2	Ru, Sh
AmcAmp	2	1	Ru
AmcAmpDxTet	4	1	Ty
AmcAmpKCf	4	1	An
AmcAmpKCfGenNeo	6	1	Ma
AmiAmcAmpGenNeo	5	1	Be
AmiAmcAmpKCfGenNeoTmp	8	1	In
Amp	1	4	De, In, Ru
AmpCfChlStr	4	1	Ne
AmpCfTetTmp	4	1	An
AmpDxTet	3	1	De
AmpK	3	2	Pa
AmpKDxGenNeoNorSxtTet	8	1	Ty
AmpKDxGenTet	5	1	En
CazCef	2	1	En
CazNeoNor	3	1	Ru
Cef	1	1	De
Cf	1	2	Be, In
Chl	1	1	Ne
ChlDxFloTet	4	1	Ty
ClNeo	2	1	Pa
DfChlFloSxtTet	5	1	Ce
DxNeoTet	3	1	De
DxTet	2	5	De, In
DxTmp	2	1	Ma
Flo	1	1	Ty
K	1	15	En, Le, Lo, ne, Pa, Ru
KCaz	2	1	Be
KCfChoStrGenNeo	8	1	Pa
KCfClDxGenNeoTetTmp	8	1	Pa
KDxNeoTet	4	1	De
KDxTet	3	1	De
KNeo	2	1	Li
KNor	2	1	Pa
Nal	1	3	Ha, Pa, Ty
NalAmcAmpKCf	5	1	Ne
NalAmcAmpKCfCazChlClDxNeo	10	1	En
NalAmcAmpKDxEnrGenNeoTetTmp	10	1	Ty
NalAmpDxStxTetTmp	6	1	Ce
NalDxNeoSxtTetTmp	6	1	En
NalDxSxtTetTmp	5	2	En
NalDxTet	3	2	Ty
NalK	2	2	Ne, Pa
NalKCipNor	4	1	Pa
NalKClStrGen	5	1	Ty
NalKDxTet	4	1	De
NalKNeo	3	1	In
NalSxtTmp	3	1	Ty
NalTet	2	1	Ty
Neo	1	7	Be, In, Ori, Pa
Nor	1	1	In
Str	1	1	Pa
Tmp	1	2	Pa, Ty
Susceptible/intermediate	0	91	An, Be, Ce, De, En, Dia, Gi, Ha, Ins, Le, Li, Lo, Ma, Mo, Ne, Oh, Or, Ori, Pa, Ru, Sp, Sf, Ty, Wo

[a] Nalidixic acid (NAL), Amoxicillin/Clavulanic Acid (AMC), Amikacin (AMI), Ampicillin (AMP), Kanamycin (K), Cephalothin (CF), Ceftazidime (CAZ), Ceftiofur (CEF), Ciprofloxacin (CIP), Chloramphenicol (CHL), Colistin (CL), Doxycycline (DX), Enrofloxacin (ENR), Streptomycin (STR), Florfenicol (FLO), Gentamicin (GEN), Neomycin (NEO), Norfloxacin (NOR), Sulfametazol + Trimethoprim (SXT), Tetracycline (TET), Trimethoprim (TMP).

[b] An = Anatum, Be = Belem, Ce = Cerro, De = Derby, En = *Enterica*, Dia = Diarizonae, Gi = Give, Ha = Hadar, In = Infantis, Le = Lexington, Li = Livingstone, Lo = London, Ma = Madelia, Mo = Montevideo, Ne = Newport, Oh = Ohio, Or = Oranienburg, Ori = Orion, Pa = Panama, Ru = Rubislaw, Sp = Saintpaul, Sf = Senftenberg, Sh = Shwazengrund; Ty = Typhimurium, Wo = Worthing.

The upper sub-basin had the lowest diversity of serovars (Fig. 5). This region had the largest area of forest. It means fewer sources of point and non-point pollution, which determines a positive environmental impact on the microbiological quality of water. Site 1 had a lower environmental pressure (dairy and agriculture) than Site 3 (dairy, broiler, swine, and agriculture), but the farm had a large area of forest, and therefore, the greatest diversity of serovars can be attributed to the presence of wildlife.

Despite Sites 5 and 6 presenting the highest number of positive samples, they showed low diversity of serovars. It can be explained by the pattern of production in these two Sites: a high presence of industrial swine and broiler production systems, therefore, genetic, nutritional, and health managements are standardized. It can indicate that the industrial livestock production means a high presence of *Salmonella* in the environment, but with low diversity.

Site 7 differed from the other Sites because it had the highest human density in the study area, with homes, a school, and a cemetery. The presence of humans and their domestic activities is also a potential source of *Salmonella* for the environment, and even in sub-basins of agricultural profile, its presence should be considered, especially in areas of human concentration, and rate of rural sanitation are low or septic tank systems are not correctly managed.

In the current study, 49.5% of the tested isolates were resistant to at least one antimicrobial, and multi-resistance occurred in 18% of isolates. The percentage of *Salmonella*-resistant isolates observed in the Pinhal catchment, 49.5%, was lower than the 83% previously found in pig's environment/animal samples in the same region area (Kich et al., 2011).

Peak et al. (2007) demonstrated a strong relationship between on-Site Confined Animal Feeding Operations (CAFO), antibiotic use, and resistance-gene abundances in surface waters at the field-scale. This relationship was verified in our study. More than 10% of isolates were resistant to ampicillin, doxycycline, nalidixic acid, neomycin, kanamycin, and tetracycline, with the last two presenting the highest resistance, 21.1% and 13.8%, respectively. Resistance lower than 1.0% was observed for ciprofloxacin and enrofloxacin.

However, antibiotic resistance is a dynamic phenomenon and the results change among different studies, samplings, and locations. For instance, Watabe et al. (2003) observed that 57.7% of the *Salmonella* isolates from pig slurry displayed antibiotic resistance to at least two antibiotics, 34.6% of isolates to three agents, and the remainder (7.7%) were resistant to four antibiotics. Our group had shown 43% of *Salmonella* isolates resistant to four or more antibiotics among 572 studied in Santa Catarina State (Kich et al., 2011). A high percentage (95%) of tetracycline resistance and 30% of multi-resistance (four or more antimicrobials) were found by Bessa et al. (2006) in *Salmonella* isolates from swine lymph nodes, tonsils, intestinal content, and pork sausages, from animals that were raised in nine farms in Rio Grande do Sul State in Brazil.

The differences in levels of antimicrobial resistance and decreasing susceptibility may be a result of the common use of prophylactic antimicrobials added to the feed for swine and broiler. An increased therapeutic antimicrobial use on dairies may also create a selection pressure for higher levels of antimicrobial resistance (Berge et al., 2008; Kemper, 2008; Kolpin et al., 2002).

The impact of antimicrobial drugs administered to animals on the resistance of microorganisms depends not only on the amount used and the type of administration, but also on animal husbandry practices, metabolism within the animal, manure handling, and storage and degradation rates. Because of this, it is imperative to obtain a better database of the productive, agricultural, and environmental managements to improve the knowledge and to determine the relationship between antimicrobials used in livestock and environmental quality.

Unfortunately, we did not have the information about antibiotic use in each farm, but we can observe that the use of prophylactic antimicrobials in swine and broiler feed was common. We also know that the use of swine, broiler, and dairy manure was done in the wrong way, without considering the nutrient balance and farms did not have a Nutrient Management Plan. These conditions increase the risk of developing resistance and are a threat to human, animal, and environmental health.

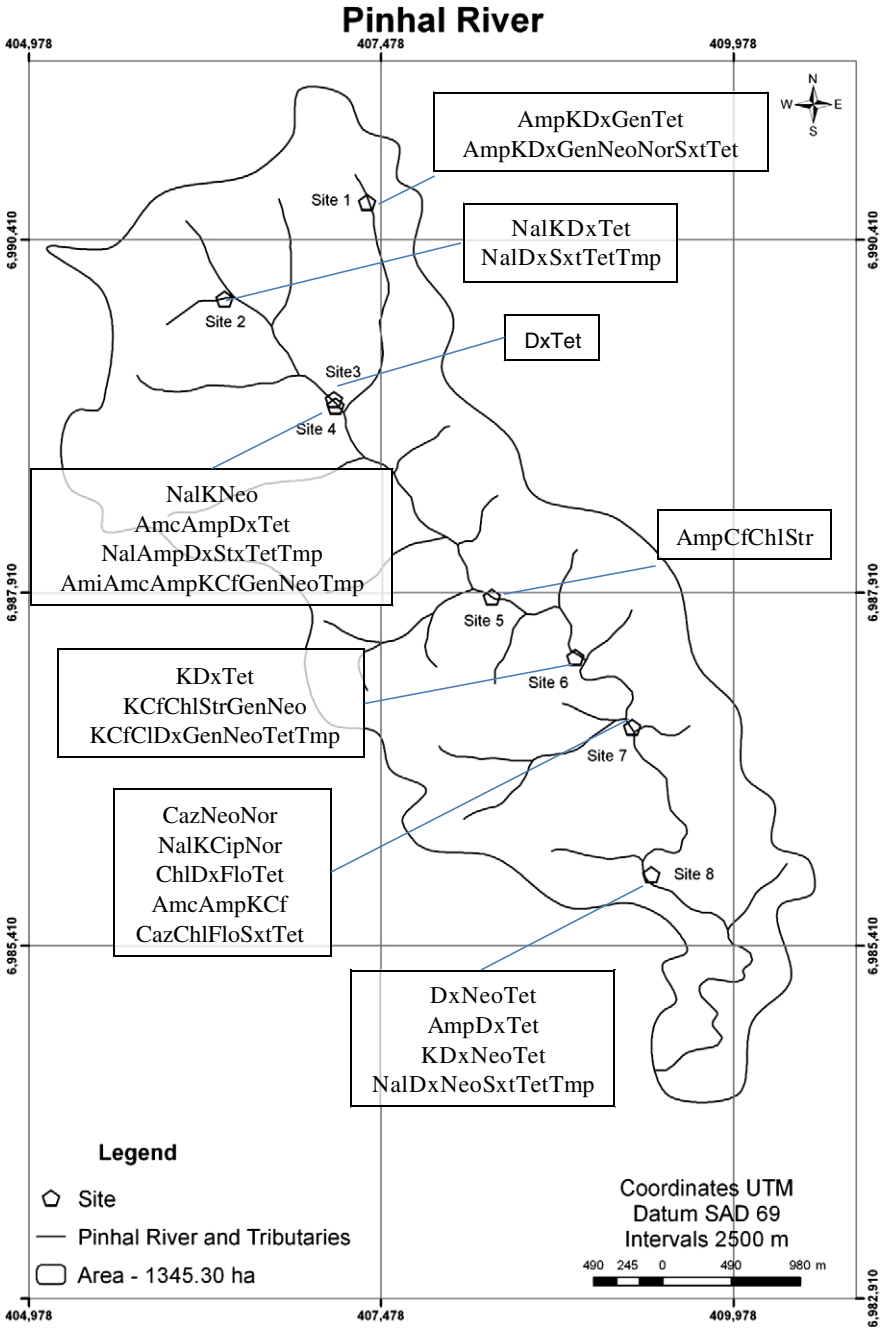

Fig. 7. Multi-resistance by sampling Site.

5. Conclusions

Salmonella was detected throughout the Pinhal catchment indicating a close interaction between animal-based agriculture, pathogen, and antimicrobial resistance.

The scenarios in the present and the future of animal production are: high animal density per area, reduced soil for the use of manure as fertilizer, and conflicts over water use. Conducting studies that will evaluate the detection of antibiotic resistance in microorganisms is of public interest, and in basins characterized by intensive animal production will aid communities, society, and governments in decision-making. These studies must be connected with animal health policies that promote the monitoring of animal operation to obtain information on the basis of farm-related data, individually, regionally, and related to individual product sectors.

One of the most important strategies must be to maintain the effectiveness of current drugs by using them responsibly, but this demands a change in attitudes to their consumption. We must invest in better education and training for veterinarians and farmers and more comprehensive information for the general public on the harm done by using antimicrobials improperly in livestock production.

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