RESEARCH PAPER

Presence of tetracycline resistant bacteria and genes in grassland-based animal production systems

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Abstract

L. López, J. Santamaría, A. Sánchez, L. Castro, and J.L. Moreno. 2012. Presence of tetracycline resistant bacteria and genes in grassland-based animal production systems. Cien. Inv. Agr. 39(3): 411-423. This study assessed the presence of tetracycline-resistant bacteria in five different grassland-based production systems dedicated to raising dairy cattle located in the Colombian Andes. Animal (ruminal fluid and feces) and environmental (soil and water) samples were evaluated, and resistant heterotrophic bacteria were found in rumen fluid, feces, soil, and runoff water samples. The resistant bacteria were isolated and identified based on the 16S rDNA region. Subsequently, they were evaluated for the presence of *tet* genes, which encode ribosomal protection proteins and membrane efflux pumps. The most frequent phyla detected were Firmicutes and Proteobacteria. The most common resistance genes found were *tet*(W), *tet*(Q), and *tet*(M). The nucleotide sequences of the genes showed no differences in bacteria isolated from environmental samples versus ruminal fluid and feces. This result suggests that the observed environmental resistance in the evaluated grasslands is the result of horizontal gene transfer from animals to the environment.

Key words: Antimicrobial resistance, cattle farms, Colombian Andes, environmental pollution, tetracycline.

Introduction

Livestock production in Latin America and the Caribbean is extensively developed, corresponding to approximately 550 million hectares of cattle farmlands (FAO, 2008). Several studies have indicated that indiscriminate management practices in these systems have played an important role in soil degradation, biodiversity losses and water and air pollution (McAlpine *et al*., 2009; Steinfeld *et al*., 2006). However, the administration of antibiotics to animals to control diseases has not been considered in environmental impact studies.

Considering that approximately 30% of Latin America and the Caribbean lands (Steinfeld *et al*., 2006) are used in livestock production systems, contamination by antibiotics that can spread through the environment, mainly through resistant bacteria and antibiotics in manure and animal

urine, is an emerging concern (Compagnolo *et al*., 2002, Winkler and Grafe, 2001). Antibiotics released into the environment can select populations of resistant organisms that, together with enteric microorganisms released in feces, can transfer resistance genes to native soil and water microorganisms through mobile genetic elements (MGEs). This contributes to the spread of resistant microorganisms in the environment (Götz and Smalla, 1997; Heuer and Smalla, 2007; Schwarz *et al*.; 2006 Sengelov *et al*., 2003; Witte, 2000), as MGEs allow genetic information to be transferred among microorganisms through transformation, transduction and conjugation processes (Black, 1999). This propagation results in an increase in the probability that resistance will be transferred to pathogenic bacteria affecting humans and animals through drinking water and agricultural products. Previous studies conducted in intensive-type animal production facilities, where antibiotics are not only used to control bacterial infections but also as growth promoters, have shown that these production systems are a source of surface and underground water contamination by antibiotics (Aminov *et al*., 2001; Heuer *et al*., 2011, Knapp *et al*., 2010, Patterson *et al*., 2007, Peak *et al*., 2007).

Generally, the antibiotic administration in extensive production systems is significantly lower than in intensive production systems, as the use of antibiotics is restricted to disease control, and they are not supplied as growth promoters. Despite the low amounts of antibiotics administered in these production systems, they may still lead to the formation of resistant reservoirs in animals (Bryan *et al*., 2004), and environmental pollution by antibiotics may also occur when animal feces and their associated microorganisms deposited on the ground. During rainy seasons, runoff and infiltrating water can become sources of resistant microorganisms contaminating the surface and groundwater (Koike *et al*., 2007). Although grasslands occupy a large area of Latin America and the Caribbean, no evaluations of these systems as a source of antibiotic contamination have been conducted in the region.

The aim of this study was to assess the presence and phylogenetic identity of tetracycline-resistant heterotrophic bacteria in soil, water and animals on livestock farms. Tetracycline was chosen for investigation because it is used worldwide due to its broad spectrum effectiveness and low cost in the market. Additionally, the presence, similarity and diversity of *tet* genes (Roberts, 2005), which are responsible for tetracycline resistance, were evaluated in bacteria isolated from environmental and animal samples to determine whether it is likely that these resistance genes are spreading into the environment.

Materials and methods

Study site

This study was conducted on five dairy farms located in a high plateau of the Western Colombian Andes known as the Altiplano Cundiboyacense (Figure 1). The farms that were sampled in this study, the Manitas, Puente Luna, Granada, Lindaraja and Alisos farms, are under extensive-type management. The diet of the animals on these farms basically depends on local grazing and is not supplied as concentrates with growth promoters. The use of antibiotics is limited only to treating sick animals, which is part of a strict management regime, as most of their milk is purchased by the dairy industry, and if traces of antibiotics are found, the product will be rejected.

Sample collection

Sampling events were carried out on each farm. Samples of soil, ruminal fluid, animal feces and water were collected. On each farm, two 10x20 m plots were established, both of which contained livestock feces. In each plot, four 10-m long transects separated by 5 m were established. Four soil samples (S), between 0 and 5 cm deep, were randomly collected from each transect. The samples from each transect were mixed to form
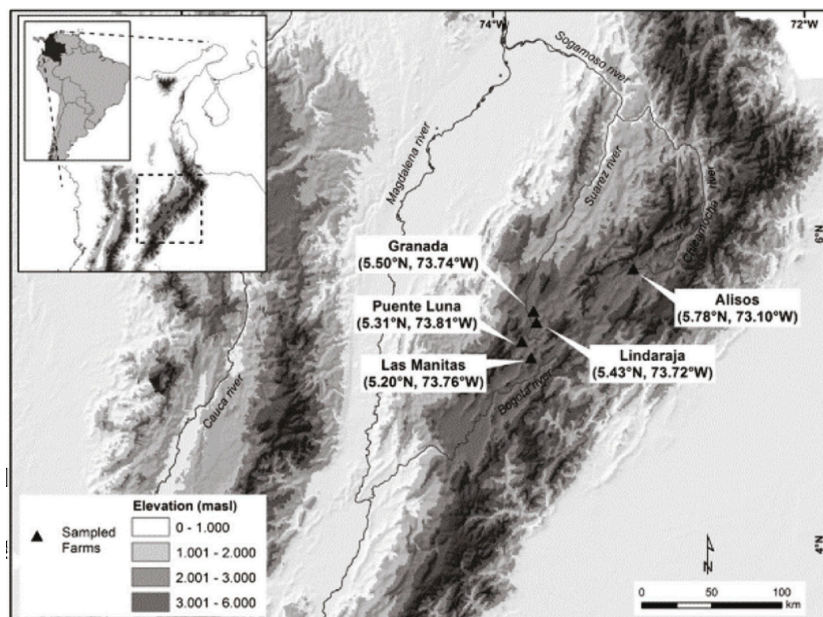
Figure 1. Locations of sampling sites in the Altiplano Cundiboyacense, Colombian Andes. The geographic coordinates for the sites are indicated in parentheses.

a composite sample. A total of eight composite soil samples were collected from each farm. Feces samples (F) were collected only from one of the plots according to the design described for soils. A sample of 50 g of fresh feces was collected at each of the sampling points in the area. The samples collected from each transect were mixed to form a composite sample. At each farm, a total of four composite feces samples were collected.

Samples of runoff, underground and animal drinking water were collected depending on their availability at the farm. Runoff water (RW) was collected from drainage ditch systems, except at Manitas, where it was collected from a pond of accumulated runoff water. To obtain these samples, 50 mL sterile containers were immersed approximately 50 cm below the water surface at five different sampling points, for a total of five samples. The animal drinking water (ADW) used at Granda, Lindaraja and Alisos is *in situ*-treated runoff water. At Puente Luna however, the animal drinking water is the same water consumed by the human population and comes from municipal treatment plants. A 100 mL sample of this water was collected from one of the drinking fountains

on each farm using a sterile container. Groundwater (GW) samples from active agricultural wells at Alisos and Manitas were collected from the valve system installed at the top of each well, allowing the water run for 30 minutes prior to filling a sterile glass container of 150 mL. Using a bovine gavage, approximately 100 mL of ruminal fluid (RF) was collected from one animal per farm, except at Manitas. All of the samples were stored at 4° C, transported to the laboratory and processed within 24 h.

Quantification of resistant bacteria

To determine the number of colony forming units (cfu) of heterotrophic resistant bacteria, 25 mL of each water sample was resuspended in 225 mL of saline solution (0.99%), and 10 g of each soil and feces samples was resuspended in 90 mL of the same solution. Counting was carried out via spreading samples on agar media at a 1:10 dilution. Media supplemented with 20 µg µL^{-1} of oxytetracycline hydrochloride, 95% HPLC (Sigma life science ref. O5875-10gG) and antibiotic free-media were used to determine the total content of cultivable

heterotrophic bacteria. Groundwater samples were placed directly onto the plates without dilution. The total contents of heterotrophic and resistant bacteria in ruminal fluid were quantified using the roll-tube method described by Holdeman and Moore (1972). Each environmental and animal sample was analyzed in triplicate. A total of 140 oxytetracycline (OTC)-resistant bacterial colonies were isolated and purified using the streak plate method for subsequent identification.

DNA extraction and PCR amplification

The DNA of tetracycline-resistant morphotypes isolated from soil, water, feces and rumen fluid samples was extracted using the Ultra Clean 15 DNA kit (Mo Bio Laboratories Inc. Carlsbad, CA, USA). A portion of the biomass of the microorganisms was transferred to a tube containing stone spheres and SDS solution using an inoculation loop. This tube was held at -20° C for 15 min and subsequently transferred to boiling water for 15 min. From this point on, the protocol was developed according to the manufacturer's instructions.

The extracted DNA was used to amplify the 16S rDNA region from the resistant colonies, whose phylogenetic identity was determined through sequencing of these amplicons. The amplification reactions were carried out using a Labnet thermocycler in a final reaction volume of 50 μL. The reaction mixture contained reaction buffer (1x), 0.2 mM each oligonucleotide, 1 U of Taq polymerase (Taq Go Flexi, Promega), 250 nM each primer, 2 mM $MgCl_2$, 400 ng of fetal bovine serum (BSA Bioline) and 1 μL of the sample DNA solution. In the amplification reactions targeting the 16S rDNA region, the universal primers 8 F-GC (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCCGCA-3 ') were used to amplify a 1,533 bp region (Loffler *et al*., 2000). The amplification program was as follows: 94° C for 4 min (1 cycle); 94° C for 50 sec, 55° C for 45 sec, 72° C for 50 sec (30 cycles); and 75° C for 7 minutes. To evaluate the presence

of genes encoding tetracycline ribosomal proteins (*tet*(M), *tet*(O), tetB (P), *tet*(Q), *tet*(W), *otr* (A)) and tetracycline efflux pumps (*tet*(A), *tet*(B), *tet*(D), *tet*(H), *tet*(J), *tet*(Z)), the following program was used: 94° C for 4 min (1 cycle); 94°C for 50 sec, X °C for 40 sec, 72° C for 30 sec (30 cycles); and 72° C for 4 min. The annealing temperatures (X) were specific for each pair of primers used. The details of the primers targeting each of the genes listed above and the annealing temperatures are described by Aminov *et al*. (2001 and 2002). For use as a positive control, 250 bp DNA fragments containing sequences aligned to *tet* genes were synthesized by DNA Technologies (Inc. San Diego). The control sequences were obtained from Aminov *et al*. (2001 and 2002).

Sequencing and sequence analysis

The PCR products from the 16S rDNA region and from the most common *tet* genes were sequenced using the DYEnamic ET Dye terminator kit (MegaBACE) in a MegaBACE 1000 sequencer (GE Amersham). The identity of the PCR products was confirmed by comparing their nucleotide sequences with the National Center for Biotechnology (NCBI) GenBank database using the BLAST tool.

Data analysis

The different types of samples collected within a farm were each taken as an experimental unit. The differences in the cfu values among the sample types from each farm were determined using the Kruskal-Wallis H test, as the data did not show a normal distribution. This test conducts multiple comparisons of the mean ranges for all groups to compare the mean cfu values for different samples to determine which of these values are significantly different. This analysis was conducted separately at each sampling site.

To determine the similarity in the bacterial phylogenetic composition between animal and

environmental reservoirs, a similarity matrix was generated using the Dice index, which was represented in a UPGMA dendrogram by the program XLSTAT 6.0 (Adinnsoft, NY USA, 2011). To determine the similarity among sequences of *tet* genes detected in strains isolated from environmental and animal samples, alignment and clustering analyses were carried out using the Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, MI, USA, 2009) and Clustal W 2.1 (European Bioinformatics Institute, Dublin, Ireland, 2008) programs.

Results

CFUs of resistant bacteria

Considering all of the farms sampled, the population of OTC-resistant bacteria represented 0.09-2.74% and 0.2-5.8% of total cfu in soils and RW, respectively, which are the types of samples subjected to the greatest exposure to animal feces and urine (Figure 2). All of the samples of ruminal fluid and feces were positive for resistant bacteria, which ranged from 0.06-1% and 0.15-3.7% of the total bacteria present, respectively (Figure 2). In the ADW and GW samples, which are less exposed to animal waste, resistant bacteria represented 0-0.8% and 0-2.9% of the total bacteria. Although the percentages of resistant bacteria were similar among the different types of samples, the numbers of resistant bacteria in the ADW and GW samples tended to be lower than in soils and RW. The cfu values for resistant bacteria in the ADW samples were significantly lower than in the RF and F samples from Puente Luna (H = 54.7, df = 4, α = 0.05, P$\leq$0.01), Granada (H = 52.2; df = 4, α = 0.05, P$\leq$0.005), Lindaraja (H = 39, df = 4, α = 0.05, P$\leq$0.002) and Alisos (H = 41.7, df = 5, α = 0.05, P$\leq$0.01). The cfu values of resistant bacteria in the GW samples are also significantly lower than in the RF and feces samples from Alisos (F = 41.7, df = 5, α = 0.05, P$\leq$0.001) and compared to the feces samples from Manitas (H = 28.45, df

= 3, α = 0.05, P$\leq$0.00008). The highest resistant bacteria contents were observed in the RF (10^5-10^6 cfu mL^{-1}) and feces (10^4-10^5 cfu g^{-1}) samples, except at Manitas (10^3 cfu g^{-1}) (Figure 2).

Bacterial composition

To assess the phylogenetic composition of the tetracycline-resistant bacteria on livestock farms within the Altiplano Cundiboyacense and compare the bacteria in the animal and environmental samples, 140 resistant isolates were obtained through OTC cultivation.

The analysis of the 16S rDNA region indicated that the isolated strains are distributed in four different groups (Table 1): 37.4% Firmicutes, 32% Proteobacteria (mainly γ-Proteobacteria), 6% Bacteroidetes and 6% Actinobacteria; 20% of the total bacteria could not be identified.
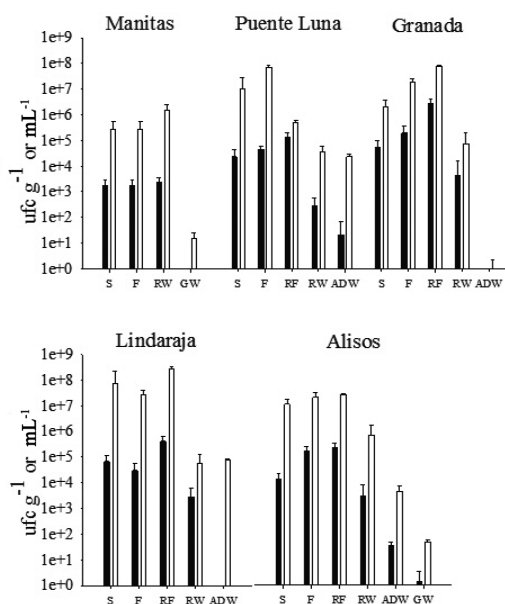

Figure 2. Numbers of heterotrophic bacteria resistant to oxytetracycline and total bacteria in environmental and animal samples. (S) Soils, (F) feces, (RF) ruminal fluid, (RW) runoff water, (ADW) animal drinking water and (GW) ground water. (■) 20 µg µL^{-1} of OTC, (□) no antibiotic.

Table 1. Phylogenetic identification of isolates resistant to oxytetracycline based on the 16S rDNA region.

Phylum	Closest sequence in GenBank	No. of isolates	Isolate source
Actinobacteria	*Arthrobacter* sp.	2	F, RF
	Leucobacter comagatae	1	ADW
	Streptomyces sp.	5	S, RW, ADW
Bacteroidetes	*Bacteroides bacterium*	1	S
	Chryseobacterium sp.	5	S, RW, ADW
	Flavobacterium johnsoniae	1	RW
	Sphingobacterium sp.	1	ADW
Firmicutes	*Bacillus* sp.	4	S, F
	Enterococcus sp.	16	S, RW, F,RF
	Exiguobacterium sp.	1	S
	Kurthia gibsonii sp.	1	F
	Lactobacillus sp.	3	RW, RF
	Paenibacillus sp.	2	S, F
	Pediococcus pentosauceus	1	RW
	Staphylococcus sp.	10	S, RW, ADW
	Streptococcus sp.	13	RF
Proteobacteria α	*Caulobacter* sp.	1	RW
Proteobacteria β	*Achromobacter piechaudii*	1	S
	Burkholderia sp.	2	S, RW
	Variovorax sp.	5	S, RW, ADW
Proteobacteria γ	*Acinetobacter* sp.	13	S, RW, ADW, F
	Aeromonas sp.	1	RF
	Dyella sp.	1	ADW
	Escherichia coli	7	S, F, RF
	Klebsiella sp.	4	S, RF
	Morganella morganii	1	RW
	Providencia sp.	2	GW
	Pseudomonas sp.	1	RW
	Serratia marcescens	3	S, ADW
	Shigella flexneri	1	F
	Stenotrophomonas sp.	2	S, RW

n = 140; it was not possible to identify 28 of the isolates.
(S) Soil, (RW) runoff water, (ADW) animal drinking water, (GW) ground water, (RF) ruminal fluid,
(F) feces.

Cluster analysis showed that the phylogenetic composition of the isolates obtained from the environmental samples (soil, RW, GW and ADW) was significantly different from the composition detected in animal samples (feces and rumen fluid) (Figure 3).

Diversity and distribution of tet genes

Among the 140 isolates, 87% were positive for the presence of at least one *tet* gene. The remaining 13% did not present any of the selected genes (Table 2). The identified genes included *tet*(M), *tet*(O),

tetB (P), *tet*(Q), *tet*(W), *tet*(A), *tet*(H), *tet*(Z) and *tet*(B). The undetected genes were *tet*(J), *tet*(D) and *otr*(A). Genes encoding ribosomal protection proteins exhibited the highest detection frequencies, representing 180 out of the 199 PCR-positive reactions for *tet* genes evaluated (Table 2). Within this group, the most frequent genes were *tet*(W),

corresponding to 33% of the reactions (67 out of 199); *tet*(Q), to 26% (52 out of 199); and *tet*(M), to 24.6% (49 out of 199).

The 140 isolates showed 28 different patterns regarding the presence of *tet* genes. The majority of isolates (29%) presented two different genes
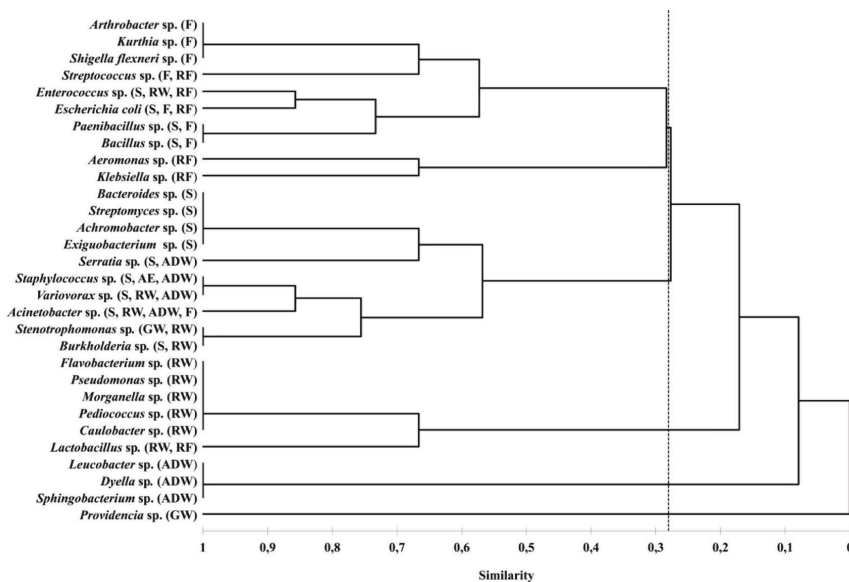

Figure 3. Cluster analysis of environmental and animal reservoirs based on the identity of bacteria isolated from (S) soil, (RW) runoff water, (ADW) animal drinking water, (GW) ground water, (RF) ruminal fluid and (F) feces. (UPGMA, Dice index).

Table 2. Results of PCR evaluation of the presence of *tet* genes in heterotrophic bacteria isolated from environmental and animal samples.

Sample type	No. of isolates evaluated by PCR	*tet* genes encoding ribosomal protection proteins					*tet* genes encoding membrane efflux pumps				
		tet(M)	*tet*(O)	tetB(P)	*tet*(Q)	*tet*(W)	*tet*(A)	*tet*(H)	*tet*(Z)	*tet*(B)	
Environmental											
S	42	9 (21)	1 (2)	1 (2)	10 (24)	14 (33)	2 (5)	1 (2)	-	2 (5)	40
RW	25	13 (52)	1 (4)	1 (4)	16 (64)	18 (72)	-	1 (4)	1 (4)	-	51
ADW	10	7 (70)	0	1 (10)	6 (60)	7 (70)	-	1 (10)	-	-	22
GW	1	-	-	-	-	1	-	-	-	-	
Animal											
RF	19	15 (79)	5 (26)	1 (5)	8 (42)	16 (84)	4 (21)	1 (5)	-	-	50
F	25	5 (20)	1(4)	-	12 (48)	11 (44)	-	-	1 (4)	5 (20)	35
		49	8	4	52	67	6	4	2	7	199

(Frequency of detection) = No. of isolates positive for a given gene/total no. of evaluated samples x 100.
(S) Soil, (RW) runoff water, (ADW) animal drinking water, (GW) ground water, (RF) ruminal fluid, (F) feces.

Table 3. Distribution of tetracycline genes among the isolates obtained from environmental and animal samples.

Patterns of *tet* genes	No. of isolates with the same pattern	%	Genera
tet(M)	6	4.3	*Enterococcus, Streptococcus, Bacillus, Variovorax, Acinetobacter, Streptomyces, Staphylococcus, Lactobacillus,* Isolate code[1] 123
tet(W)	12	8.6	*Bacillus, Streptomyces, Variovorax, Chryseobacterium, Klebsiella, Strepnotrophomonas, Enterococcus, Staphylococcus, Acinetobacter, Leucobacter,* Isolate code[1] 23
tet(Q)	10	7.1	*Arthrobacter, Burkholderia, Chryseobacterium, Enterococcus,* Isolates code[1] 36, 60, 86
tet(Z)	1	0.7	Arthrobacter
tet(B)	1	0.7	*Escherichia*
tet(M),(W)	15	10.7	*Acinetobacter, Chryseobacterium, Enterococcus, Escherichia, Kurthia, Lactobacillus, Morganella, Streptococcus, Streptomyces, Variovorax,* Isolate code[1] 14
tet(M),(Q)	7	5	*Acinetibacter, Bacillus, Caulobacter, Pseudomonas, Staphylococcus,* Isolate code[1] 42, 87
tet(M),(O)	1	0.7	*Streptococcus*
tet(Q),(W)	12	8.6	*Acinetobacter, Enterococcus, Escherichia, Exiguobacterium, Paenibacillus, Strepnotrophomonas, Streptomyces, Variovorax,* Isolate code[1] 10, 22, 54, 84, 89
tet(O),(B)	1	0.7	*Streptococcus*
tet(Q),(B)	1	0.7	*Escherichia*
tet(W),(B)	1	0.7	*Shigella*
tet(W),(A)	1	0.7	*Enterobacter*
tetB(P),(W)	2	1.4	Streptomyces, Sphingobacterium
tet(M),(O),(W)	1	0.7	*Acinetobacter*
tet(M),(Q),(W)	12	8.6	*Acinetobacter, Chryseobacterium, Dyuella, Enteroccocus, Flavobacterium, Pediococcus, Staphylococcus, Streptococcus,* Isolate code[1] 21
tet(M),(W),(B)	1	0.7	*Acinetobacter*
tet(M),(W),(A)	2	1.4	*Escherichia, Klebsiella*
tet(M),(W),(H)	2	1.4	Acinetobacter, Paenobacillus
tet(M),(W),(Z)	1	0.7	*Enterococcus*
tet(M),(W),(B)	1	0.7	*Escherichia*
tet(W),(A),(H)	1	0.7	*Escherichia*
tet(M),(O),(Q), (W)	4	2.9	*Streptococcus*
tet(M),(Q),(W),(A)	1	0.7	Streptococcus
tet(M),B(P),(W),(A)	1	0.7	*Aeromonas*
tet(M),B(P),(O),(W)	1	0.7	*Staphylococcus*
tet(M),(Q),(H),(B)	1	0.7	*Enterobacter*
tet(M),(Q),(W), (H)	1	0.7	*Staphylococcus*
(-)	39	27.9	Isolates with any *tet* gene detected

[1]The isolates indicated by their code were not identified.

(Table 3), and 6.4% of the isolates exhibited a maximum of four different *tet* genes.

Origin of resistance in the environment

The *tet* gene amplification products were sequenced to confirm their identities, and the sequences of the most frequently detected genes were used to compare the genes amplified from environmental samples with those of animal origin.

BLAST analysis confirmed that the sequences corresponded to the selected genes, and the results of the alignment analysis for the most common genes, *tet*(W), *tet*(Q) and *tet*(M), were as follows. The *tet*(W) and *tet*(M) sequences showed no differences among the isolates that were positive for these genes (data not shown). Regarding the *tet*(Q) gene sequences, the alignment analysis showed three different genotypes, exhibiting differences regarding the nucleotides at position 32, where thymine is replaced by cytosine, and position 65, where thymine is replaced by adenine compared to the gene sequence from *B. thetaiotaomicron* used as a positive control. The *tet*(Q) gene similarity analysis grouped the analyzed sequences according to the nucleotides at positions 32 and 65 and not based on the type of sample. In Figure 4, it can be seen that group one is formed by identical sequences that present differences in the two positions (32 and 65); in group two, all of the sequences exhibit an adenine at position 65; and in the third group, all of the sequences are identical to the positive control. It can also be noted in Figure 4 that the three groups present *tet* sequences detected in bacteria from both environmental and animal origins.

Discussion

The animals from the extensive production systems evaluated in this study presented high levels of OTC-resistant bacteria. This conclusion is based on the finding that feces and rumen fluid presented

resistant bacteria values of between $3x10^4$ and $3x10^6$ cfu g^{-1} at most of the farms sampled, except at Manitas, where the manure value reached $1.7x10^3$ cfu g^{-1}. Therefore, animal waste released into the environment is loaded with OTC-resistant bacteria. Environmental samples of soil and RW, which are subjected to high exposure to animal waste, showed resistant bacteria populations of between $1.6x10^3$ and $6.5x10^4$ cfu g^{-1} in soil and $2.4x10^3$ and $7.6x10^3$ cfu mL^{-1} in water, except for in RW at Puente Luna, where the population did not exceed $2.7x10^2$ cfu mL^{-1} (Figure 2). It is well known that in general, samples of animal origin will exhibit a large number of bacteria, as the
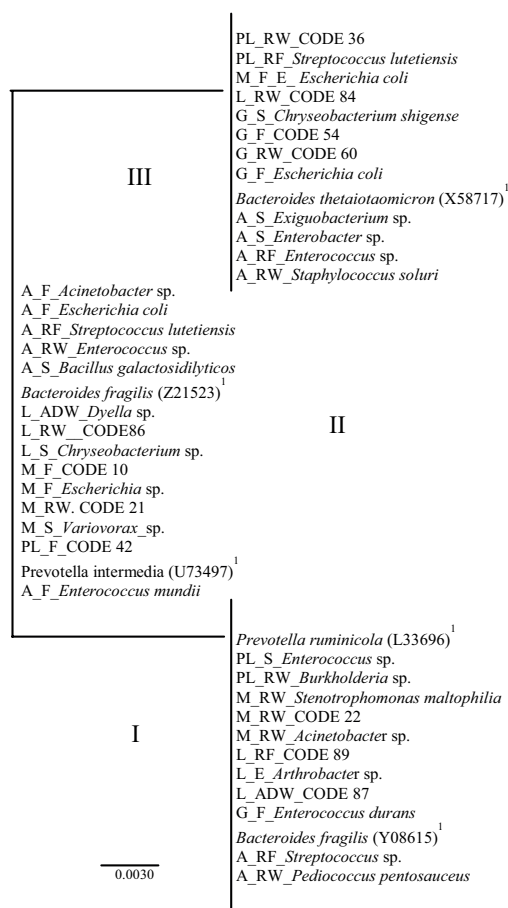

Figure 4. Phylogram showing the genetic distances among *tet*(Q) gene sequences (neighbor-joining). Sampled farms = (M) Manitas, (PL) Puente Luna, (G) Granada, (L) Lindaraja and (A) Alisos. Type of sample = (S) soil, (RW) runoff water, (ADW) animal drinking water, (RF) ruminal fluid and (F) feces. [1]Gene accession number in the GenBank database.

physicochemical conditions of inside animals favor the growth of microbial populations. In contrast, the bacteria living in the environment are subjected to adverse temperatures and limited availability of nutrients. The concentration of microorganisms in the environment therefore tends to be lower (Maier *et al*, 2009).

The ADW and GW samples, which are less exposed to animal waste, presented smaller bacterial population sizes, between 0 and $9x10^0$ cfu mL^{-1}, which is the result of the chemical treatments applied to runoff water, municipal plant water treatment and the groundwater not receiving a high load of infiltration water. The presence of resistant bacteria in ADW, which is stored in drinking fountains, occurs because this water is completely exposed to the environment. Therefore, any type of particle can be deposited in it, and fecal contamination was often detected in this water source. Although the cfu values in animal drinking water were low, these results indicate that animals are constantly drinking contaminated water. The only GW-isolated bacterium identified belonged to the *Providencia* genus, within the family of enteric bacteria. This bacterium may have reached the groundwater through runoff water infiltration. It is worth noting that the population of resistant bacteria in the environment may be greater than that reported in this study, as the applied technique only quantifies cultivable microorganisms.

As a result of fecal contamination spreading in the environment, genera such as *Enterococci*, *Klebsiella* and *Escherichia*, which are typical inhabitants of animal intestinal tracts, were isolated from soil and water samples (Table 1 and Figure 3). However, most of the tetracycline-resistant bacteria isolated from environmental samples, such as *Chryseobacterium*, *Variovorax*, *Streptomyces*, *Burkholderia*, *Caulobacter* and *Sphingobacterium*, are environmental native microorganisms. Although some of the isolated resistant bacteria genera were present in both the environment and in animals, the two types of

reservoirs tended to exhibit significantly different phylogenetic compositions, as shown in Figure 3.

It is likely that the observed resistant bacteria isolated from the environment are the product of horizontal transfer from animals into the environment. While it is not possible to determine the origin of resistance on the evaluated farms using molecular techniques, it can be determined whether there is resistant gene flow from one compartment to another (animal/environment). The method for tracking the flow of a gene based on nucleotide sequence similarity has been widely applied, as shown in studies by Jansen *et al*. (1998), Scott *et al*. (2000) and Aminov *et al*. (2001). The sequence similarity among resistance genes detected in animal and environmental samples in this study, despite the fact the two compartments host different microbial communities (Figure 3), provides evidence of horizontal transfer through mobile genetic elements between these two compartments.

The data obtained in this study do not suggest that the gene transfer flows from the environment to animals because if this were the case, genes from soil-native and antibiotic-producing bacteria, such as *otr*(A) (Aminov and Mackie, 2007), would have been detected in animal samples, or new sequences of the resistance genes *tet*(W), *tet*(M) and *tet*(Q) would have been found. These phenomena were not observed in our results. Instead, all of the nucleotide sequences identified in the environment exactly match the resistance gene sequences reported in the literature (Aminov *et al*., 2001; Billington *et al*., 2002; Flórez *et al*.; 2006 Koike *et al*., 2007; Spigaglia *et al*., 2008). This finding and the fact that the most common genes detected in animal samples (*tet*(W), *tet*(M) and *tet*(Q)) were also the most common genes detected in environmental samples suggests that on these farms, resistance is flowing from animals to the environment.

In the Altiplano Cundiboyacense, the most frequently detected genes were those encoding

ribosomal protection proteins. Genes encoding tetracycline efflux pumps were not frequent. In contrast, in the United States, the *tet*(A) and *tet*(B) genes, which encode efflux pumps, are the most frequently detected in cattle feces (Sawant *et al.*, 2007). In Japan, genes encoding efflux pumps are also the most frequent in agricultural environments (Kobayashi *et al.*, 2007). Only Yang *et al.* (2010) has reported higher frequencies of genes encoding ribosomal protection proteins in cattle farms under intensive production.

In this study, it was found that most of the obtained isolates exhibited one or two *tet* genes, which has also been reported in previous studies (Bryan *et al.*, 2004; Tao *et al.*, 2010). The most common genes were *tet*(W), *tet*(Q) and *tet*(M), which were widely distributed in the isolates obtained from the different types of samples (Table 3). The wide distribution of *tet*(W), *tet*(Q) and *tet*(M) among different genera is consistent with the genetic mobility reported for these genes (Roberts, 2005).

In conclusion, extensive livestock production systems exhibit both animal and environmental reservoirs of resistant bacteria, and it is very likely that the presence of resistant bacteria in soil and water is due to gene flow mainly from animal waste. As the occurrence of genes encoding OTC resistance was remarkable on the sampled farms, where antibiotic use is restricted, it is advisable to also conduct this type of assessment in the industrial production systems located in the region. In these systems, such resistance assessments generally focus on bacteria isolated from animals.

Acknowledgements

Resumen

L. López, J. Santamaría, A. Sánchez, L. Castro y J.L. Moreno. 2012. Presencia de bacterias y genes de resistencia al antibiótico tetraciclina en sistemas de producción ganadera, basados en pasturas. Cien. Inv. Agr. 39(3): 411-423. En este estudio se evaluó la presencia de reservorios ambientales y animales de bacterias resistentes a la oxitetraciclina (OTC) en cinco sistemas de producción extensivo dedicados a la cría de ganado lechero y localizados en el Altiplano Cundiboyacense. Los tamaños poblacionales de las bacterias heterótrofas resistentes, estimados en muestras ambientales (suelo y agua) y animales (líquido ruminal y estiérco) mediante técnicas de cultivo, fueron notables especialmente en muestras de líquido ruminal, estiércol, suelo y agua de escorrentía. Bacterias resistentes fueron aisladas, identificadas mediante la región 16S ADNr y evaluadas para la presencia de genes *tet* que confieren resistencia a la tetraciclina y que codifican para proteínas de protección ribosomal y proteínas membranales de eflujo. Los filos más comunes dentro de las 140 cepas aisladas

fueron Firmicutes y Proteobacteria dentro de las cuales se encontraron géneros típicos tanto del ambiente como del tracto intestinal. Los genes de resistencia más comunes fueron *tet*(W), *tet*(Q) y *tet*(M) y la comparación entre sus secuencias no muestran diferencias entre genes de bacterias aisladas del ambiente y bacterias del tracto digestivo de los animales, lo que sugiere que los reservorios de resistencia en el ambiente son el resultado de la transferencia horizontal de genes desde las bacterias resistentes de los reservorios animales.

Palabras clave: Andes colombianos, fincas ganaderas, resistencia antimicrobiana, contaminación ambiental, tetraciclina.

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