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IDENTIFICATION OF ANTHROPOGENIC IMPACT ON NATURAL HABITATS BY ANTIMICROBIAL RESISTANCE QUANTIFICATION IN TWO NEOTROPICAL WILD CATS AND THEIR GEOSPATIAL ANALYSIS

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ABSTRACT: Human activities such as habitat degradation and fragmentation threaten biodiversity in Neotropical areas. This work proposes an analytical methodology to identify natural areas in Central America with anthropogenic impact, analyzing the presence of antimicrobial resistance genes (ARGs) in accordance with their theoretical relationship with human-related activities. Sixteen ARGs were quantified in feces of different individuals of 13 jaguars (*Panthera onca*) and 13 pumas (*Puma concolor*) in three conservation areas in Costa Rica by real-time PCR. At least one ARG was detected in all samples. Of the ARGs encoding tetracycline resistance, the most frequent were *tet*Q and *tet*Y (85% and 69%, respectively). The sulfonamides (*sul*I and *sul*II; 69% each), phenicols (*cat*I and *cat*II; 19% and 54%, respectively), and quinolones (*qnr*S; 12%) were also detected. The presence of human settlements, livestock farms (pigs, cattle, and poultry), roads, human health centers, flood zones, and rivers were identified within each area to generate an index of human activity. We found no difference between the presence of ARG by roads, agricultural activities, and human settlements ($P>0.05$). However, *tet*W showed higher percentages with porcine and bovine farms; both *tet*Y and *tet*W were more frequent in jaguars than in pumas. Of concern is that many of the most contaminated samples were taken from national parks, such as Braulio Carrillo and Tortuguero, where animals should not have direct contact with humans.

Key words: Antimicrobial resistance, Costa Rica, human activities, Neotropical wild cats, *Panthera onca*, public health, *Puma concolor*.

INTRODUCTION

One of the emerging global threats to biodiversity is antimicrobial resistance resulting from the broad use of antibiotics in livestock and agriculture (Qiao et al. 2018). Over the last several decades, this use of antibiotics has led to positive selection for resistant bacterial strains, negatively affecting human and nonhuman animal health (O'Neill 2014; World Health Organization 2014; Qiao et al. 2018). Antimicrobial resistance is expected to become a leading cause of worldwide human mortality by 2050, surpassing cancer and traffic accidents (O'Neill 2014; World Health Organization 2014). Additionally, antimicrobial resistance is expected to affect global economies negatively from increased treatment costs, hospitalizations, and antibiotic compound research and development (Tzoc et al. 2004; Ventola 2015).

Antibiotics may be released into the environment through treatment plants, sewage sludge, sediments, and water (surface, underground, and residual). For example, residual

waters run freely in rivers through ecosystems, where susceptible bacterial communities can acquire antibiotic resistance by incorporating mobile genetic elements such as antimicrobial resistance genes (ARGs; Daughton and Ternes 1999). This selection occurs more rapidly in environments where the use of antimicrobials is higher (van de Sande-Bruinsma et al. 2008), such as those surrounded by livestock activity.

Wild fauna may be directly exposed to antibiotics (and resistant bacteria) by drinking contaminated water and through the consumption of contaminated prey (Tzoc et al. 2004; O'Mahony et al. 2006; Rodriguez et al. 2006; Sarmah et al. 2006; Qiao et al. 2018). Carnivores, given their position in the trophic chain and habitat requirements (Arroyo-Arce et al. 2014; Petracca et al. 2014), are good indicators of ecosystem health. They therefore may serve as valuable models to detect the presence of ARGs and their potential dissemination in the environment (Cevidanes et al. 2020; Sacristán et al. 2020).

Recently, a possible relationship between landscape anthropization degree and the prevalence of ARGs in feces from South American wild cats has been studied (Cevidanes et al. 2020; Sacristán et al. 2020). A similar finding was determined in urban pigeons in the most urbanized, populated, and economically active region of Costa Rica (Blanco-Peña 2017). However, whether these contaminants are present in wild fauna in this country is unknown.

Pumas (*Puma concolor*) and jaguars (*Panthera onca*) are the two largest carnivores in Costa Rica. Wild cats may use a wide variety of habitats (Escobedo Grandez 2011), including fragmented landscapes (Crooks and Sanjayan 2006) and areas adjacent to or with moderate levels of anthropogenic activity. Therefore, jaguars and pumas inhabiting such areas may present a higher frequency of resistance gene–carrying bacteria than those inhabiting more remote areas (Daughton and Ternes 1999; Tzoc et al. 2004; Ventola 2015). Our objective was to identify whether the presence of ARGs in the top neotropical predators, jaguars and pumas, can be related to anthropogenic activity levels.

MATERIALS AND METHODS

Study area

Samples were collected in three conservation areas managed by the Costa Rican government through the National System of Conservation Areas (SINAC) and on private lands. The Arenal-Huetar Norte Conservation Area, specifically the Maquenque National Wildlife Refuge, is surrounded by many rivers and lagoons. Historically, the most effective production activity in the area has been livestock (Chassot et al. 2009).

The Central Conservation Area (CCA) includes La Selva Biological Station, Alberto Manuel Brenes Biological Reserve, Braulio Carrillo National Park, the Quetzales National Park, and the Cordillera Volcánica Central Forest Reserve. This region has high mountains with dense forests and many rivers, where livestock is present (Chassot et al. 2009; SINAC 2019).

The Tortuguero Conservation Area (ACTo) contains the Barra del Colorado National Wildlife Refuge and the Tortuguero National Park. The area has rivers, swamps, lowland forests with various drainages, and hill forests (Castillo et al. 2000; Ling 2002; Chassot 2006; Polidoro et al. 2009; SINAC 2019).

All these areas are surrounded by human activities characterized by high use of agrochemicals, fertilizers, deviation of river courses, and water pollution (Jong 2001; Wo-Ching et al. 2001). These characteristics made them an attractive model considering the relationship between the presence of ARGs, proximity of human activities, and proximity of large wild cats.

Scat samples

Wild cat scat samples were collected opportunistically by noninvasive methods (without capturing the animals) for a previous study between 2011 and 2012, with collaboration of researchers and organizations that contributed to felid conservation in situ (Soto-Fournier 2014). Therefore, the scat sampling was aimed to obtain especially wild felid species. In some cases, a trained dog was used to localize felid scat. When possible, the field collector recorded species identification by size of scat or near scrapes and tracks, so each sample could be given a confidence label or at least was listed as a "possible" identification.

Samples were dried with silica beads and stored at room temperature or frozen at –20 C. Species identification was conducted at the Global Felid Genetics Program at the Sackler Institute for

Comparative Genomics at the American Museum of National History (New York, New York, USA). Species-specific primers amplified regions of five mitochondrial genes: cytochrome *b* (H15149, Farrell-R: Kocher et al. 1989; Farrell et al. 2000), 12S (L1085, H1259: Kitano et al. 2007), 16S (L2513, H2714: Kitano et al. 2007), 16Scp (16Scp-F, 16Scp-R: Kitano et al. 2007), and adenosine triphosphate-6 (ATP6-DF3, ATP6-DR2: Chaves et al. 2012). Sequences were edited by Sequencher, version 5.0 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and Geneious Pro, version 6.1.5. (Biomatters Ltd., Auckland, New Zealand). Moreover, they were aligned to an in-house reference database compiled for carnivore species. Sequence similarity among species was assessed by constructing a phylogenetic tree with the neighbor-joining method to infer the origin of the samples. Fragments were sequenced in both directions. For this study, jaguar and puma samples were used; geographic coordinates of samples were available. All samples were analyzed according to Costa Rican regulations (permits R-018-2015-OT-CONAGEBIO, R-019-2015-OT-CONAGEBIO, R-021-2015-OT-CONAGEBIO, R-004-2017-OT-CONAGEBIO, and R-005-2017-OT-CONAGEBIO).

Antibiotic resistance gene detection

Detection of antibiotic resistance genes was conducted in the Center for Animal Health Research, part of the National Institute for Agricultural and Food Research and Technology, Ministry of Economy and Competitiveness, Madrid, Spain.

We extracted DNA directly from all fecal samples with the QuickGene DNA Tissue Kit S (Fujifilm, Japan) following the manufacturer's instructions. We amplified 16S rRNA genes to detect the presence of bacteria in each sample following the methodology as described by Doi and Arakawa (2007). We considered a valid sample as those in which the 10-fold dilution showed a cycle threshold (Ct) of <30.

The ARGs analyzed were selected as representatives of six antibiotic groups: tetracyclines, chloramphenicols, sulfonamides, quinolones, vancomycin, and methicillin. We detected ARGs by real-time PCR with SYBR Green, although a TaqMan probe was used to detect the gene *mec*A (Francois et al. 2003; Jiang et al. 2013; Marti and Balcázar 2013).

We approximated the number of ARG copies with the formula (Sacristán et al. 2020)

$$\log(\text{ARG\%}) = 2 + 0.33(\text{Ct}_{16\text{SrRNA}} - \text{Ct}_{\text{ARG}}),$$

where $\text{Ct}_{16\text{SrRNA}}$ is the cycle threshold for the bacterial determination, Ct_{ARG} is the cycle threshold for each gene, and 0.33 is the mean slope for all the genes tested. We expressed results in $\log_{10}$ of the hypothetical percentage of bacteria that each gene presents for the percent load of ARG. Because the exact date the felines defecated the samples is unknown, this formula allows a relative quantification by the number of bacteria present in each sample. Our results were highly correlated with previously published data (Xie et al. 2016). Samples were also classified into "multiresistant microbiome" (at least three ARGs encoding resistance to different groups of antimicrobials) and "non-multiresistant microbiome" ARGs (Karczmarczyk et al. 2011; Sacristán et al. 2014).

Geographic analyses

We analyzed the microbiologic results along with the geographic and physical characteristics of the areas. We considered the jaguar home range as a diameter of 7 km around the collection site for each sample, as defined from previous studies ($\sim$40 km^2; Rabinowitz and Nottingham 1986). No data were available for pumas, so the same range was used by default. The presence of human settlements, livestock farms (pig, cattle, and poultry), roads, human health centers, flood zones, and rivers was identified within each area as possible contact sources. The relationship between the normalized concentration of ARGs and human activities was examined from the information generated.

To determine the spatial distribution of ARG prevalence, we used the software for geographic information system analyses, QGIS (2.2.0-Valmiera; QGIS.org 2014) and ArcGIS (version 10.3; Esri, Redlands, California, USA), with the following layers: geographic for Costa Rica, protected areas, biological corridors, forest coverage, hospitals, topographic relief, river layer 1:200,000, villages, and land use (Ortiz and Soto 2014). Data related to livestock production (bovine, porcine, avian) was generously provided by the National Animal Health Service (SENASA, Heredia, Costa Rica) from a census performed in 2014.

Statistical analysis

We used the R Statistical Computing Environment and the ggplot2 package to visualize the ARG prevalence results in a heat map (Wickham 2016; R Core Team 2019). Additionally, a normality test was performed on the 14 ARGs. Because of their normal distribution, parametric tests were applied. A principal component analysis was performed by the FactoMineR package (Lê et al. 2008) to summarize the database matrix of the 14 resistance genes and to analyze which

genes had the greatest weight per feline species and which genes were correlated. Additionally, two multivariate analysis of variance (MANOVA) tests were performed for the first and second principal component axes. The first was to identify a difference between ARG amounts (response variables) and species (explanatory variables). The second was to find the possible contact sources according to the geographic analyses and explanatory variables.

RESULTS

Fecal samples from 13 jaguars and 13 pumas were included in this study (Fig. 1).

Antibiotic resistance gene detection

All samples were positive for at least one ARG. Antibiotic resistance genes encoding tetracyclines were found in 25/26 samples (96%), and at least one gene was quantified in each sample (Fig. 2). The most frequent among the genes were *tet*Q and *tet*Y (85% and 69%, respectively). Genes encoding resistance to sulfonamides (*sul*I and *sul*II) were found at 69% each. Additionally, phenicols (*cat*I and *cat*II), and quinolones (*qnr*S) were found at 19%, 54%, and 12%, respectively. In samples from jaguars, the highest percentage corresponded to *tet*Q (6.1×10^{-1}%), followed by *tet*W (1.5×10^{-1}%), whereas in samples from pumas, the highest averages were *tet*Y (1.2×10^{1}%) and *tet*B (1.0×10^{1}%). The ARGs *van*A and *mec*A were not detected (Table 1 and Fig. 2).

The sample with the highest ARG number (14 genes) was from a jaguar from the Tortuguero National Park, located in the ACTo. According to the geographic analyses, it was surrounded by nearly 16 porcine farms. In contrast, the sample with the smallest number of ARGs (one gene) was from a puma sample found in a mountain range of La Selva Biological Station, CCA, very close to human towns (<1.6 km). La Selva Biological Station and Maquenque National Wildlife Refuge showed the lowest mean number of ARGs (three). The Tortuguero National Park had a mean of eight, from 10 animals (Table 2).

According to the results obtained by principal component analysis (Fig. 3), the first component explained 21.6% of the total variance, positively related to *cat*I and *tet*Q and negatively related to the rest of the ARGs. For this first axis, the eigenvalues indicated that *tet*A and *tet*Y were the genes with the greatest weight. The second axis explained 17.4% of the variance. On this axis, it was observed that most of the genes encoding resistance to tetracyclines and phenicols were positively related, whereas sulfonamides were negatively related. For the second axis, according to the eigenvalues, the *cat*II and *tet*W genes had the greatest weight (Table 3).

The percentages of *tet*Y, *tet*W, *sul*I, and *cat*I according to species were different (MANOVA = Wilk lambda 0.558; $F_{3,169}$; P=0.028). The jaguar samples showed a higher percentage of ARGs for *tet*Y and *tet*W, whereas *tet*A and *cat*II maintained similar percentages (Fig. 4). A total of 48% of the samples carried a multiresistant microbiome, with 41% positive for resistance to three families of antimicrobials and 7% positive for resistance to four families.

Geographic analyses

In the Braulio Carrillo National Park, CCA, we found high *tet* concentrations, especially *tet*Y and *tet*B, shown by two pumas from two low-lying areas. Also, high and very high percent loads for *cat*I and *cat*II were found mainly in a puma sample surrounded by mature and secondary forests, rivers, roads, villages, one cattle farm, and low flood areas (see Supplementary Material Fig. S1). One of the samples with high *sul* values was collected from a puma in the Braulio Carrillo National Park. The sampling point was a mature forest area close to a river and a cattle farm, no nearby villages, and low flooding incidence. For samples with high *tet* presence, 6/10 were collected in the Tortuguero National Park in ACTo and a private livestock farm close to it. Both sites were <1 km from one health center, 12 pig farms, and 67 bovine farms. The sample with a high value of *sul*II came from a ranch located close to ACTo and belonged to a jaguar that preyed on cattle (Soto-Fournier 2014). The *qnr*S was only found in three

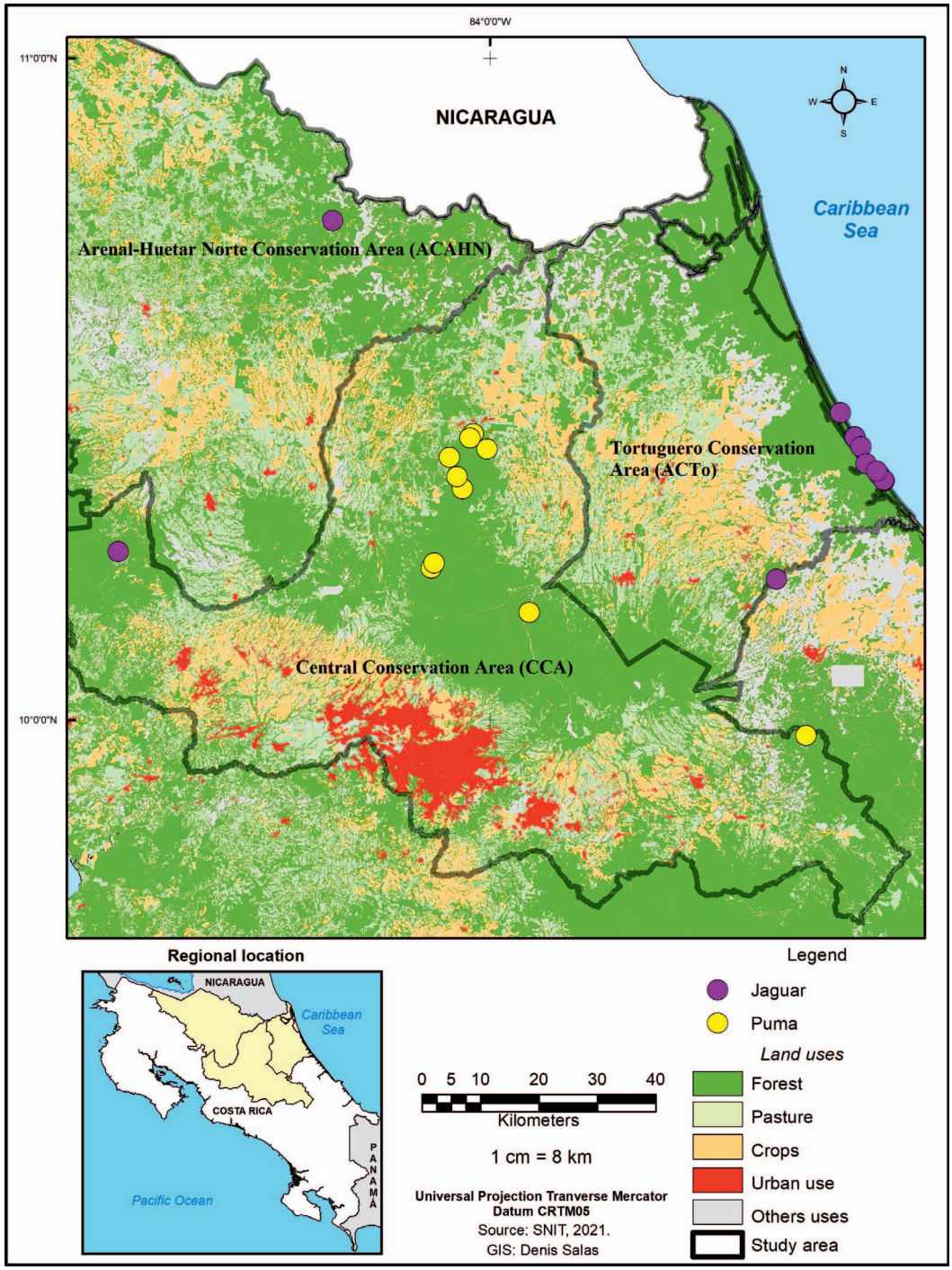

FIGURE 1. Map of the conservation areas in Costa Rica showing land use and where scat samples of jaguars (*Panthera onca*, circles in purple) and pumas (*Puma concolor*, circles in yellow) were collected.

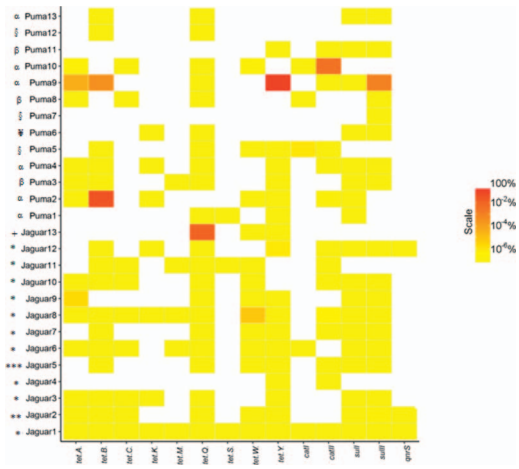

FIGURE 2. Percent load of antimicrobial resistance genes (ARGs) in fecal samples of jaguars (*Panthera onca*) and puma (*Puma concolor*) from Costa Rica. The scale indicates the relative concentration of each gene. The white color represents the absence of ARGs. Samples were collected from *Tortuguero National Park (ACTo), **Siquirres (ACTo), ***Alberto Manuel Brenes Biological Reserve (CCA), [+]Maquenque National Wildlife Refuge (ACAHN), [α]Braulio Carrillo National Park (CCA), [β]Forest Reserve (CCA), [§]La Selva Biological Station (CCA), [¥]Quetzales National Park (CCA).

jaguars. The highest concentration of this ARG was detected in a female jaguar feeding on cattle (Soto-Fournier 2014) from a farm located far away from national parks. It also had intermediate to high concentrations of *sul*II, *sul*I, *cat*I, and *cat*II (a multiresistant microbiome). The other two positive samples were collected in the Tortuguero National Park but showed lower values of ARGs. Land use around these sampling points included intervening pastures, secondary forests, roads, villages, and flood zones, and 67 bovine and 12 pig farms were within a 1-km radius.

Possible contact sources

According to the MANOVA test, there was no difference between ARGs and the presence of roads ($P>0.05$) and livestock activities ($P>0.05$). However, *tet*W showed higher percentages near, specifically, porcine and bovine farms (see Supplementary Material Fig. S1). The presence of human settlements, human health cares, flood zones, and rivers was not significant ($P>0.05$) because they were present in all (or almost all) the sampling

TABLE 1. Number of copies of antimicrobial resistance genes in fecal samples of jaguars (*Panthera onca*) and pumas (*Puma concolor*) from Costa Rica.[a]

	Jaguar			Puma				
Gene	Min	Max	Mean	Min	Max	Mean	Total mean	% positive
*tet*A	7.3×10^{-5}	1.3×10^{0}	9.5×10^{-2}	3.8×10^{-3}	2.6×10^{0}	2.5×10^{-1}	1.7×10^{-1}	50
*tet*B	5.2×10^{-6}	8.1×10^{-2}	6.1×10^{-3}	3.9×10^{-5}	1.3×10^{2}	1.0×10^{1}	5.0×10^{0}	65
*tet*C	5.5×10^{-6}	5.1×10^{-4}	9.5×10^{-5}	6.4×10^{-5}	6.3×10^{-2}	4.9×10^{-3}	2.4×10^{-3}	35
*tet*K	9.9×10^{-7}	2.1×10^{-4}	2.7×10^{-5}	2.6×10^{-5}	1.7×10^{-2}	1.3×10^{-3}	6.6×10^{-4}	27
*tet*M	2.2×10^{-6}	4.6×10^{-2}	4.7×10^{-3}	n.d.	9.7×10^{-5}[b]	7.5×10^{-6}	2.5×10^{-3}	19
*tet*Q	3.9×10^{-7}	8.5×10^{0}	6.1×10^{-1}	9.8×10^{-6}	2.6×10^{-1}	2.3×10^{-2}	3.3×10^{-1}	85
*tet*S	2.1×10^{-7}	4.4×10^{-7}	4.7×10^{-8}	n.d.	1.4×10^{-2}[b]	1.1×10^{-3}	5.3×10^{-4}	12
*tet*W	4.4×10^{-6}	1.8×10^{0}	1.5×10^{-1}	2.9×10^{-4}	4.2×10^{-1}	3.3×10^{-2}	9.4×10^{-2}	50
*tet*Y	1.7×10^{-5}	8.7×10^{-1}	8.4×10^{-2}	2.9×10^{-5}	1.5×10^{2}	1.2×10^{1}	5.6×10^{0}	69
*cat*I	n.d.	2.6×10^{-4}	1.9×10^{-5}	5.1×10^{-5}	9.2×10^{-1}	7.9×10^{-2}	3.8×10^{-2}	19
*cat*II	2.0×10^{-5}	5.8×10^{-1}	5.9×10^{-2}	1.7×10^{-4}	6.8×10^{0}	5.6×10^{-1}	3.0×10^{-1}	54
*sul*I	2.1×10^{-5}	5.7×10^{-2}	7.6×10^{-3}	1.4×10^{-4}	9.8×10^{-2}	1.0×10^{-2}	8.8×10^{-3}	69
*sul*II	1.7×10^{-5}	4.2×10^{-1}	7.0×10^{-2}	3.0×10^{-4}	6.0×10^{0}	4.8×10^{-1}	2.7×10^{-1}	69
*qnr*S	6.6×10^{-7}	6.2×10^{-3}	4.4×10^{-4}	n.d.	n.d.	n.d.	2.3×10^{-4}	12
*van*A	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0
*mec*A	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0

[a] Min = minimum; Max = maximum; n.d. = not detected.

[b] Detected only in one sample.

TABLE 2. Comparison of the mean of positive fecal samples to antimicrobial resistance genes (ARGs) of jaguars (*Panthera onca*) and pumas (*Puma concolor*) from different sampling areas in Costa Rica.

Sampling area	Conservation area[a]	Species	Number of individuals	$\bar{x}$ ARGs
Tortuguero National Park	ACTo	Jaguar	10	8
Siquirres	ACTo	Jaguar	1	9
Alberto Manuel Brenes Biological Reserve	CCA	Jaguar	1	7
Braulio Carrillo National Park	CCA	Puma	6	6
Forest Reserve	CCA	Puma	3	5
La Selva Biological Station	CCA	Puma	3	3
Quetzales National Park	CCA	Puma	1	4
Maquenque National Wildlife Refuge	ACAHN	Jaguar	1	3

[a] ACTo = Tortuguero Conservation Area; CCA = Central Conservation Area; ACAHN = Arenal-Huetar Norte Conservation Area.

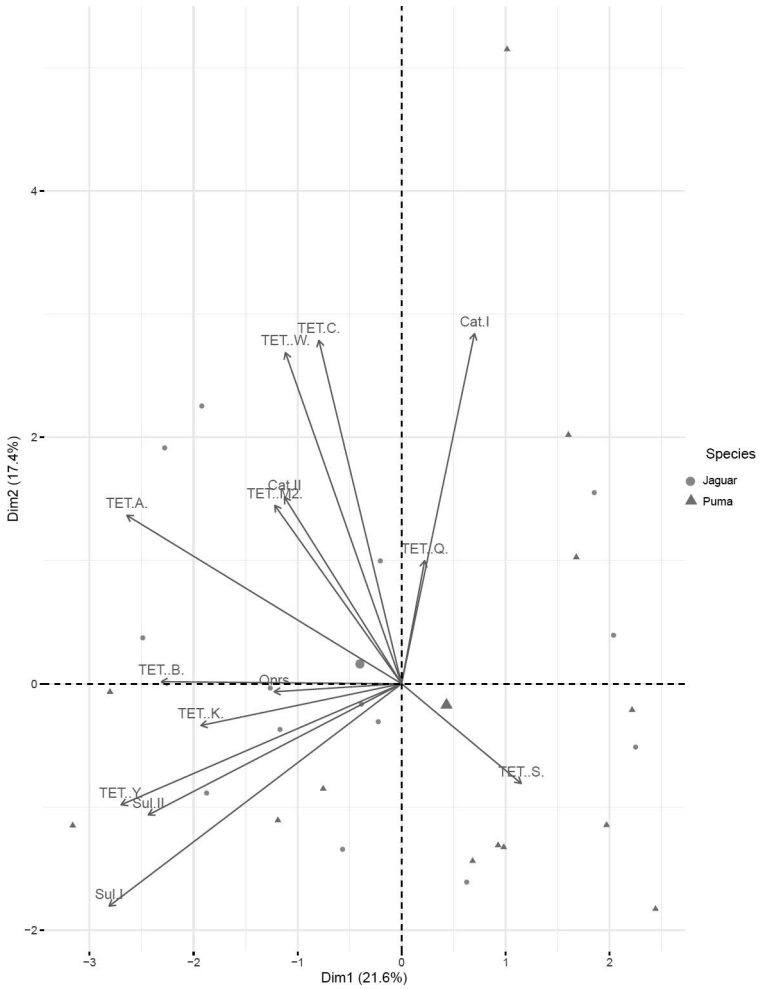

FIGURE 3. Principal component analysis biplot for antimicrobial resistance genes in scat samples from jaguars (*Panthera onca*) and pumas (*Puma concolor*) from Costa Rica.

TABLE 3. Eigenvalues of principal component (PC) analysis for antimicrobial resistance genes (ARGs) of jaguars (*Panthera onca*) and pumas (*Puma concolor*) from Costa Rica. The genes with the highest weight are in bold.

Gene	PC1	PC2	PC3
*tet*A	**0.48118**	0.1542	0.30879
*tet*B	0.36707	0.04641	**−0.4305**
*tet*C	0.06722	0.22913	0.31592
*tet*K	0.13381	−0.0214	−0.1484
*tet*M	0.10436	0.08865	0.19695
*tet*Q	0.00208	0.14385	0.33025
*tet*S	−0.0562	−0.0402	−0.0017
*tet*W	0.2086	**0.58359**	0.22536
*tet*Y	**0.4724**	−0.1804	−0.1135
*cat*I	−0.0463	0.33183	0.09428
*cat*II	0.2366	**0.43935**	**−0.5355**
*sul*I	0.36536	−0.2962	0.03648
*sul*II	0.36897	−0.3554	0.29256
*qnr*S	0.05314	−0.0255	0.05867

areas. Thus, there were no treatments for their presence and absence.

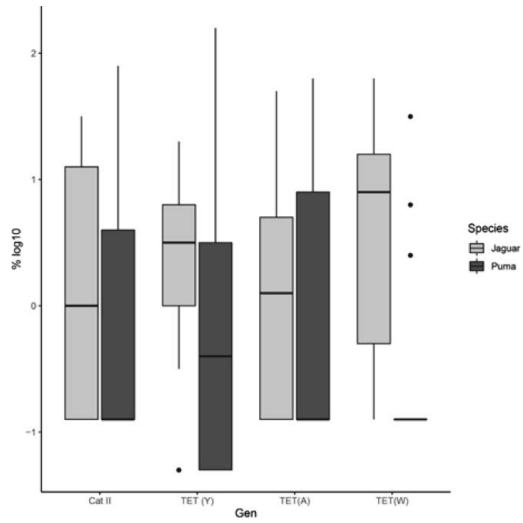

FIGURE 4. Percentage of the most important resistance genes, according to principal component analysis, found in scat samples from jaguars (*Panthera onca*) and pumas (*Puma concolor*) in Costa Rica. The *x*-axis represents the antimicrobial resistance genes and the *y*-axis the percentage of each resistance gene in common logarithm. The horizontal black lines represent the mean, the thin vertical lines represent the maximum and minimum values, and the black dots represent outliers.

DISCUSSION

In this article we provided information on the acquisition of ARGs by wild cats in a Central American country. The use of two top predators, jaguars and pumas, as bioindicators, in conjunction with the geographic and physical analyses of the area, indicate high-risk zones for acquisition of bacteria-containing ARGs. Use of passively collected scat samples, genetically identified to species and individual, provided useful data.

The ARGs analyzed were selected as representatives of six antibiotic groups important in both humans and livestock: tetracyclines, chloramphenicols, sulfonamides, quinolones, vancomycin, and methicillin. Our frequent detection of *tet* genes was expected because tetracyclines are one of the most commonly used antibiotic groups in agricultural and livestock production (Gutiérrez et al. 2010; Suzuki 2010; de la Cruz et al. 2014; Peng et al. 2015). Interestingly, high concentrations of *tet* genes were found in wild cat samples from national parks (i.e., Braulio Carrillo and Tortuguero), where lower occurrence might have been expected. Nevertheless, although these are protected areas, they are surrounded by livestock farms, towns, health centers, rivers, roads, and, in the case of Tortuguero, periodic flooding.

The percentage of ARGs encoding for phenicols was low compared with *tet* genes. The use of chloramphenicol in livestock production has not been allowed in the country since 1997 (Poder Ejecutivo 1997). However, it is not impossible that it may be used illegally (Angulo et al. 2004) or that related antimicrobials (de la Cruz et al. 2014), such as florfenicol, may be inducing resistance in bacteria.

The percentage of positive samples to *sul* genes was intriguingly higher than those reported in pigeons from public parks in the country (Blanco-Peña 2017). The low price of sulfonamides makes their use frequent in lower-income countries (Suzuki and Hoa 2012), being identified even in poultry-based

fertilizers (Zhou et al. 2017). These kinds of food supplements may be used in livestock production systems, and wild cats can acquire these genes when they feed on domestic livestock.

Although the *qnr*S gene was found only in three jaguars, their fecal samples came from one location close to livestock farms. Because these farms are medium-sized, it could be possible that they do not have adequate conditions for waste management because of their relatively high cost and complexity. However, to test this hypothesis, future investigations must consider analyzing different samples, such as effluents, from these kinds of farms.

The home range and movement behavior of jaguars and pumas may vary among species, individuals, sex, region, and habitat quality (De Angelo et al. 2011; Morato et al. 2016). Here, we considered a hypothetical area of 40 km^2 around the collection site for each sample, but this value varies among studies—most of them performed in Brazil with larger natural areas than in Costa Rica (Salom-Pérez et al. 2007; Cavalcanti and Gese 2009; Morato et al. 2016).

One might expect that pumas and jaguars in national parks would have low concentrations of ARGs. However, our findings were contrary to this expectation. Possible reasons include: 1) human activities surround these protected areas, 2) both wild cat species sometimes hunt cattle from the surrounding areas, and 3) pumas and jaguars may drink water from rivers that run through different land uses. For example, Tortuguero National Park (ACTo) is in the lower basin, so its main rivers run through towns and farms in the upper and middle basins. However, this situation may not necessarily apply to all samples obtained in CCA. Some samples came from places where surface and groundwater are theoretically unaffected by human activities.

Areas dominated by livestock activities showed higher percentages of ARGs, which may indicate a constant exposure. Wild cats, such as the jaguar and puma, come into sporadic contact with human beings, usually when they wander into farms or use human-made paths and trails in the forest (Sáenz and Carrillo 2002). It is also likely that these wild cats may have acquired resistant bacteria by drinking water, especially considering the overall annual runoff, the average annual rainfall, and the presence of at least one river within the 40-km^2 range established around each sample (Calvo 1990). Because top predators such as jaguars and pumas may acquire bacteria harboring ARGs from different sources, they are good bioindicators of environmental contamination.

According to our results, all individuals sampled in national parks included in this survey had been exposed to bacteria containing ARGs. It is of concern that scat samples from animals with theoretically reduced contact to antimicrobials showed multiresistance microbiomes.

Because of the small number of samples and the little information available about wild cat home range sizes in different areas of Costa Rica, it is not easy to establish a causal association between ARGs and human activities or other environmental factors. Although the difficulty of obtaining samples from wildlife in the tropics is enormous, their analyses provide valuable information about threats typically not included in ecosystem protection plans in any country. Moreover, the fact that puma samples were obtained from locations near the coast is valuable data to consider in future studies.

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