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Research article

Antimicrobial-resistant genes in feces from otters (*Lontra longicaudis*) within the Peñas Blancas river basin, Costa Rica

Aurora Guizado-Batista [a], Andrea Porres-Camacho [a], Seiling Vargas-Villalobos [b], Manuel Cortez-Martínez [a], Rodolfo Umaña-Castro [c], Carolina Sancho-Blanco [c], Frank Solano-Campos [c], Francisco Quesada-Alvarado [b,h], Manuel Spínola-Parallada [e], Alexander Madrigal-Mora [f], Adonay Jiménez-Serrano [c], Joshua Vargas-Calvo [c], Jenny Villalobos-Sequeira [d], Kari Brossard Stoos [g], Kinndle Blanco-Peña [b,*]

[a] *Universidad Nacional, Costa Rica. Posgrado Regional en Ciencias Veterinarias Tropicales (PCVET), Costa Rica*
[b] *Universidad Nacional, Costa Rica. Instituto Regional de Estudios en Sustancias Tóxicas (IRET), Costa Rica*
[c] *Universidad Nacional, Costa Rica. Laboratorio de Análisis Genómico (LAGen), Escuela de Ciencias Biológicas, Costa Rica*
[d] *Universidad Nacional, Costa Rica. Instituto de Estudios Sociales en Población (IDESPO), Costa Rica*
[e] *Universidad Nacional, Costa Rica. Instituto Internacional de Conservación y Manejo de Vida Silvestre (ICOMVIS), Costa Rica*
[f] *Instituto Costarricense de Electricidad (ICE), Costa Rica*
[g] *Department of Health Sciences & Public Health, Ithaca College, Ithaca, NY 14850, United States*
[h] *Programa de Doctorado en Medicina de la Conservación, Facultad de Ciencias de la Vida, Universidad Andrés Bello, Santiago de Chile, Chile*

A R T I C L E I N F O

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A B S T R A C T

Antimicrobial resistance poses a growing threat to human health, yet its implications for wildlife remain a subject of ongoing research. River otters inhabiting the Peñas Blancas River face exposure to various anthropogenic activities in their habitat, potentially leading to the accumulation of antibiotic-resistant genes (ARGs) with unknown consequences for their health. This study aimed to identify specific ARGs in otter feces from this river basin, employing quantitative polymerase chain reaction (qPCR), DNA sequencing of ARGs, and phylogenetic analysis techniques. Over the period from 2019 to 2022, we collected 102 fecal samples from otters through the Peñas Blancas River watershed, spanning its upper and middle basins. We assessed the bacterial presence via the 16S rRNA gene through qPCR analysis and screened for 12 ARGs. Sequences of 16 ARG-positive samples were subsequently analyzed using Maximum-likelihood-base taxonomic placement. In total, 56 samples tested positive for the 16S rRNA gene, with 24 exhibiting at least one ARG. Notably, three samples showcased a "multi-resistance microbiome". qPCR analyses identified seven distinct ARGs: *tetB* (in 26.8 % of the samples), *sulI* (21.4 %), *sulII* (21.4 %), *qnrS* (10.7 %), *tetQ* (8.9 %), *tetW* (7.1 %), and *tetA* (3.6 %). Phylogenetic analysis confirmed the taxonomic association of all detected ARGs, which were compared with The Comprehensive Antibiotic Resistance Database. Our findings underscore the importance of comprehending the spread of ARGs in wildlife populations, with river otters serving as potential sentinels for ARG dissemination. Moreover, they highlight the potential impact of anthropogenic activities on the health of aquatic ecosystems, emphasizing the need for proactive measures to mitigate antimicrobial resistance in natural environments.

* Corresponding author.
E-mail addresses: kblanco@una.ac.cr, kblanco@una.cr (K. Blanco-Peña).

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1. Introduction

Antimicrobial resistance (AMR) is the ability of infectious microorganisms to avoid and survive the chemical components of pharmaceuticals used to kill or control their growth [1]. Antibiotic-resistance genes (ARGs) confer antibiotic resistance in bacteria [2]. Bacteria can acquire AMR in different forms: intrinsic (interaction with natural-produced antibiotics), acquired (mutation and horizontal gene transfer [HGT]), and adaptive (transient resistance by interacting with specific environmental signals) [3]. Mobile genetic elements (MGE), such as plasmids, transposons, integrons, integrative conjugative elements, and genomic islands, which play a crucial role in HGT, mediate the capture, accumulation, and dissemination of ARGs [4].

AMR is a natural adaptation phenomenon, but human activities have an increasing impact on the development and spreading of ARGs [5–7]. For instance, livestock is a significant source of environmental ARGs [8], receiving four times the amount of drugs and antibiotics by humans for growing and prophylaxis treatment [9], such as penicillin, quinolones, tetracyclines, sulfonamides, and others [10]. The increase in pesticide use contributes to pests developing broader tolerance, thus, promoting acquired and adaptive resistance in bacteria. This phenomenon is exacerbated by pesticides sharing similar chemical components with antibiotics, such as tetracyclines and streptomycin [11]. Resistance acquisition through HGT via MGE is favored in dense communities of bacteria, like those found in livestock digestive system, pasture areas, barns, crops' soil and sewage waters [3,10–13]. Therefore, aquatic environments have been proposed as predominant ARGs reservoirs and transmission routes as they are easily accessible for anthropogenic activities [2,12].

When wildlife comes in contact with water streams with contaminated residues from anthropogenic activities, they could become vectors and sentinels for ARGs dispersal in their ecosystems [14–16]. The presence of ARGs in Costa Rican wildlife has been linked to their proximity to anthropic activities, including their potential impact on the health of the animals and the condition of their environment, as well as on public health [17–20].

River otters (*Lontra longicaudis*) are exposed to contaminated water sources and prey and thus they have been employed as bioindicators to assess the occurrence and dispersion of ARGs in aquatic environments [21,22]. These mustelids exhibit varying degrees of interaction with diverse anthropogenic activities occurring throughout the Peñas Blancas River basin [23,24]. Therefore, this study performed a non-invasive sampling method to ascertain the presence of ARGs in their feces.

The utilization of quantitative polymerase chain reaction (qPCR) for identifying specific genes streamlines the monitoring process of ARGs presence and abundance in otters' feces, providing insights into their potential impact on ecosystemic health [17,19,20,25,26]. Also, a phylogenetic analysis helped to confirm the use of qPCR as a reliable and easier technique for ARGs detection in fecal samples [17,18,26–28].

This pioneering study used qPCR and taxonomic placement using phylogenetic analysis to assess the presence of ARGs in *L. longicaudis* from the Peñas Blancas River watershed. The aim was to elucidate the dispersion of resistance genes in the aquatic ecosystem and their potential association with human settlements and activities along the river. The insights gained can aid stakeholders in identifying possible contamination pathways, mitigating the impacts of AMR on natural environments, and reducing its potential effects on public and wildlife health.

2. Materials and methods

2.1. Study area

The Peñas Blancas River is a rural river located in the Costa Rican northern region (10°15′-10°31′N, 84°28′-84°46′ W). It constitutes part of the protected areas "Bosque Eterno de los Niños" and "Reserva Biológica Monteverde" [29]. Its forest coverage includes premontane humid forests, with temperatures between 18 and 24 °C year-round [30,31]. Human activities around the river basin include agriculture (e.g., coffee, ornamental plants, fruits, tubers, forestry), livestock (e.g., cattle, pig, poultry, aquaculture), as well as tourism [23,24,32].

2.2. Sampling design and collection

Sampling sites through the Peñas Blancas River watershed were selected according to a previous study regarding river otters' habitat preference in this region [33]. These sites were designed as upper basins (where the "Bosque Eterno de los Niños" is found and little anthropic activity happens) middle and lower basins (where most human activities occur), as was established per preliminary reconnaissance before sample collection.

Sampling occurred from 2019 to 2022 during the dry (December to March), rainy (May to October), and transition seasons (April and November) by traversing the river until otters' feces were found. Each fecal sample was collected in a sterile and labeled vial with sterile gloves. Every vial included a sample code, date, and GPS coordinates. Storing was done at around 4 °C in the field and at −20 °C at the camp base until transported to the Instituto Regional de Estudios en Sustancias Tóxicas, Universidad Nacional (IRET-UNA), where they were stored at −80 °C until DNA extraction. All samples were collected and analyzed according to Costa Rican regulations (permits R-CM-UNA-002-2022-OT-CONAGEBIO, R-CM-UNA-008-2022-OT-CONAGEBIO, and R-CM-UNA-002-2023-OT-CONAGEBIO).

2.3. ARGs detection and analysis

DNA extraction was performed with the QIAmp-DNA Stool Minikit (QIAGEN®), following the manufacturer's instructions. Distilled water was used for negative controls to confirm the absence of cross-contamination during the extraction. A polymerase chain reaction (PCR) was performed to corroborate that the feces belonged to *L. longicaudis*, through taxonomic analysis of sequences of the mitochondrial control region, utilizing the primers ProL (5′-CACCACCAACACCCAAAGCT-3′) and DLH (5′-CCTGAAGTAAGAACCA-GATG-3′) [34–36].

The 16S rRNA gene, an essential taxonomic marker for bacterial communities [37], confirmed the presence of bacteria in all samples (pure and 10-fold dilution of DNA extractions). 16S-positive samples were counted when their threshold cycle (Ct) was under 25. All 16S-positive samples were analyzed to confirm the presence of ARGs. Table 1 shows all 12 ARGs selected for this study, considering their relation with prominent antimicrobial families used in anthropogenic activities [8,10,11], and previous studies done in Costa Rica and other Latin American countries [17,20,26–28]. A sample was considered ARG positive if the Ct was less than 30. However, if the Ct was close to 30, the sample was sequenced to confirm positivity. The percentage charge of an ARG was calculated through the formula:

$$\log 10(\mathrm{ARG\%}) = 2 + 0,33^{*}(\mathrm{Ct16s} - \mathrm{CtgenARG})$$

where Ct16s was the threshold cycle for bacterial determination, CtgenARG corresponded to the threshold cycle for each gene, and the value 0.33 was the mean slope for all the genes tested [17,17,26,38]. The results were expressed in log10 of the hypothetical percentage of bacteria each gene presented for the ARGs percentage load. All qPCRs were performed in a CFX96 Touch Real-Time PCR Detection System, using SYBR Green® [39].

PCR conditions were optimized per primer pair in the MacVector software. Primer pairs are in table 1S. Controls for *dfrA12*, *catII*, *erm(B)*, *qnrS*, *tetB*, *tetQ*, *tetW*, and *tetY* were synthesized by Integrated DNA Technologies as gblocks, double stranded DNA fragments. ARG control sequences were truncated between 4 and 45 nucleotides after the primer binding sites to not amplify the entire ARGs. A multi-resistant strain was used as a positive control for $bla_{\mathrm{CTX\text{-}M}}$, *sulI*, *sulII*, and *tetA*.

The 16S rRNA gene and ARGs quantitative PCRs were improved using the specificity curves obtained from the CFX Maestro Software 2.0 (Version: 5.0.021.0616) for Bio-Rad CFX Real-Time PCR Systems. Other authors have described the validation criteria used (Broeders et al., 2014; Svec et al., 2015). The results are shown in Fig. 1S from the supplemental data.

Multi-resistant microbiomes were identified according to Blanco-Peña et al. (2017) definition. It consisted of a fecal sample that presents at least three ARG encoding resistance to different groups of antimicrobials. This classification aids in distinguishing between “multi-resistant microbiomes” and “non-multi-resistant microbiomes".

2.4. Phylogenetic analysis

Sanger sequencing of ARGs PCR products from 16 ARG-positive samples was carried out (Macrogen Inc., Korea). Samples for each

Table 1
Mechanism of resistance, reference and antibiotic-resistant genes selected for river otter feces analysis in the Peñas Blancas River watershed. Genes are clustered according to their antibiotic family.

Antibiotic Family	Gene	Mechanism of resistance	Forward and Reverse Primers	Reference
Beta-Lactams	***bla*** $bla_{\mathrm{CTX\text{-}M}}$	Hydrolysis	CTATGGCACCACCAACGATA ACGGCTTTCTGCCTTAGGTT	[40]
Diaminopyrimidines	***dfrA12***	Target protection enzymes	GAGCTGAGATATACACTCTGGCACT GAGCTGAGATATACACTCTGGCACT	[41]
Phenicols	***catII***	Inactivating enzymes	GATTGACCTGAATACCTGGAA CCATCACATACTGCATGATG	[42]
Macrolides	***erm*(B)**	Ribosomal methylation	CCGAACACTAGGGTTGCTC ATCTGGAACATCTGTGGTATG	[43]
Quinolones	***qnrS***	Target protection enzymes	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	[44]
Sulfonamides	***sulI***	Ribosomal protection	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	[45]
	sulII	Ribosomal protection	TCCGGTGGAGGCCGGTATCTGG CGGGAATGCCATCTGCCTTGAG	[45]
Tetracyclines	***tetA***	Efflux pump	TCAATTTCCTGACGGGCTG GAAGCGAGCGGGTTGAGAG	[46]
	tetB	Efflux pump	CGCCCAGTGCTGTTGTTGTC CGCGTTGAGAAGCTGAGGTG	[47]
	tetQ	Ribosomal protection	AGAATCTGCTGTTTGCCAGTG CGGAGTGTCAATGATATTGCA	[48]
	tetW	Ribosomal protection	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	[48]
	tetY	Efflux pump	ATTTGTACCGGCAGAGCAAAC GGCGCTGCCGCCATTATGC	[48]

confirmed ARG were selected according to their Ct values (<25). The ARG sequences were used as queries to search for highly similar full-length sequences in the Comprehensive Antibiotic Resistance Database, CARD v3.2.9 [49] using BLASTn in Geneious Prime 2019.2.3. These full-length ARG sequences were used again as queries to search CARD for full-length sequences that had more than 65 % identity and 60 % coverage, as a cut-off to only include sequences from the same antimicrobial resistance gene family in the phylogenetic analysis. Then, to validate that the sequences obtained correspond to the expected AMR genes families and to infer their phylogenetic relationships, datasets were aligned using MUSCLE v5 [49], and the removal of poorly aligned regions was carried out with TrimAl v1.4 [50]. Maximum likelihood (ML) phylogenetic trees were inferred with IQ-TREE v2.3 [51], employing ModelFinder [52] to select the best model of nucleotide substitution according to the Bayesian information criterion. The best fit models obtained from each AMR gene family phylogenetic tree where: GTR + F + G4 in quinolones (*qrnS*), TPM3u + F for sulfonamides (*sulI*, and *sulII*), and TVM + F + I + G4 with tetracyclines (*tetA*, *tetB, tetQ*, and *tetW*). Branch supports were assessed with an ultrafast bootstrap approximation [53,54] with 1000 bootstrap replicates. Within the quinolones, a total of twenty-seven *Qnr* sequences were used, for the sulfonamides five *sul* sequences and one *farA* sequence (membrane fusion protein that is part of the farAB efflux pump) were included, and in the tetracyclines twenty *tet* and two *fusA* sequences (elongation factor G) were included.

3. Results

In total, 102 samples were obtained and processed, from which 56 (55 %) showed a Ct under 25 for 16s rRNA bacterial load. Of the positive samples, three (5 %) came from the upper river basin, and fifty-three (95 %) from the middle; no fecal samples from the lower basin were obtained (Fig. 1).

Twenty-four samples (43 %) were positive for at least one ARG, and seven genes were detected. The most common gene was *tetB* (26.8 %), followed by *sulI* (21.4 %), *sulII* (21.4 %), *qnrS* (10.7 %), *tetQ* (8.9 %), *tetW* (7.1 %), and *tetA* (3.6 %). Table 2 shows all resistance genes found and the number of positive samples detected according to their location, compared to their minimum and maximum values from log10 percentage quantification results.

The correlation analysis revealed significant inter-family relationships within these data. For instance, *tetB* exhibited a notably stronger positive correlation with *sulI*, while *sulII* displayed correlations with *tetA*, *qnrS*, *tetW*, and *sulI* (Fig. 2). Nevertheless, in contrast to findings from other studies conducted in the country, these results appear less consistent, suggesting a prior notion that ARGs presence varies based on regional disparities in human activities and substance use [18–20].

Three samples presented multi-resistant microbiomes: PEB-1 (*qnrS, tetB* and *sulI*) PEB-7 (*qrnS, tetB, sulI and sulII*), and PEB-8 (*qnrS, tetQ, tetW, sulI*, and *sulII*); all located in the middle basin. However, sample 16-PBPP2, from the upper basin, presented four ARGs (*tetA, tetB, sulI*, and *sulII*) despite being classified as a non-multi-resistant microbiome. All 24 ARG-positive fecal samples can be viewed in table 2S from supplemental data.

The phylogenetic circular topology (Fig. 3) shows a molecular validation by phylogenetics analysis of the ARGs found in

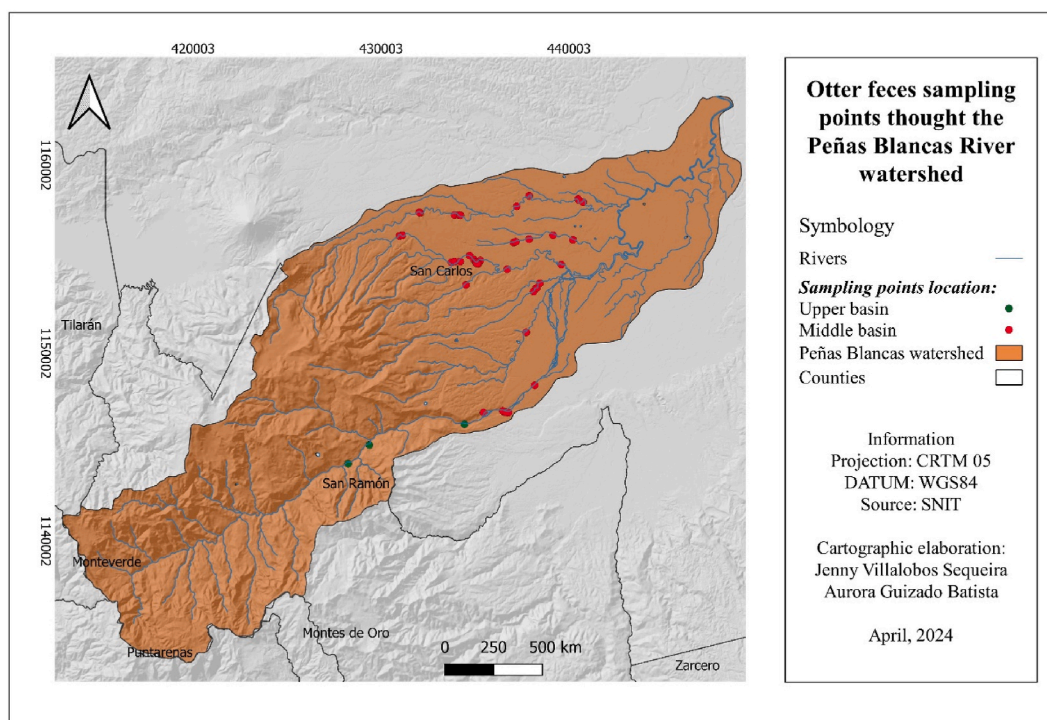

Fig. 1. Sampling points with river otter feces positive for 16s rRNA qPCR through Peñas Blancas River watershed.

Table 2
Antibiotic resistance genes detected in fecal samples from *Lontra longicaudis* at Peñas Blancas river basin. Minimum (Min) and maximum (Max) values, number (#) and percentage (%) of positive samples obtained according to their location.

ARGs	Upper basin (n = 2)				Middle basin (n = 22)			
	Min	Max	Positives		Min	Max	Positives	
			#	%			#	%
qnrS	n.d.	n.d.	–	–	−3.00	−0.09	6	27
tetA	−0.49		1	50	−0.77		1	5
tetB	−0.91	−0.82	2	100	−2.28	0.96	13	59
tetQ	n.d.	n.d.	–	–	−1.57	0.83	5	23
tetW	n.d.	n.d.	–	–	−2.41	−0.14	4	18
sulI	−0.81		1	50	−2.12	0.58	11	50
sulII	−1.56		1	50	−2.75	−0.25	11	50

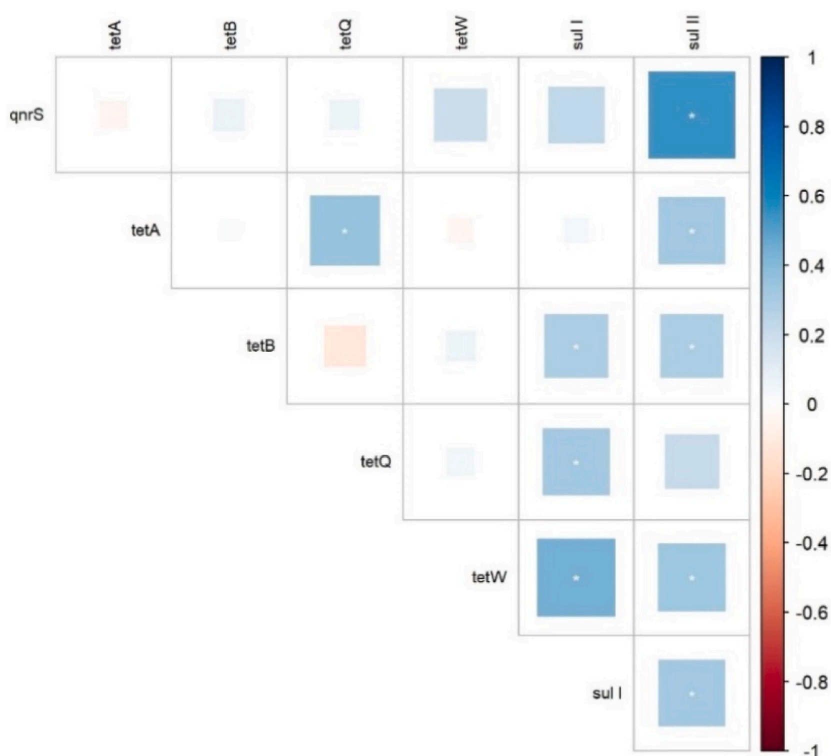

Fig. 2. Correlation among resistance genes identified in River Peñas Blancas basin otter feces. The square size indicates the strength of the relationship between variables, the color signifies whether the relationship is positive or negative, and the asterisk indicates significance.

environmental otter fecal samples and their respective association with reported plasmid-encoded genes that confer resistance to sulfonamides (types *sulI* and *sulII*), tetracycline resistance (TCR) genes, for example *tetA*, *tetB*, *tetQ*, *tetW* and plasmid-mediated quinolone resistance (PMQR) genes of the type *qnrS*.

4. Discussion

Throughout the Peñas Blancas River, seven genes responsible for resistance to quinolones (*qrnS*), tetracyclines (*tetA*, *tetB*, *tetQ*, and *tetW*), and sulfonamides (*sulI* and *sulII*) were detected in 24 river otters' feces samples. Interestingly, pharmaceutical products and metabolites belonging to all three of these antibiotic families (quinolones, tetracyclines, and sulfonamides), and beta-lactams had been found in Costa Rican wastewaters and rivers near highly populated areas [55,56]. We did not find *bla*$_{\text{CTX}}$ (beta-lactam), *dfrA12* (diaminopyrimidines), *catII* (phenicol), nor *erm(B)* (macrolide) in our samples.

Two (8 %) of the ARG-positive samples were collected in the upper basin, and 22 (92 %) came from the middle basin. All three multi-resistance samples were from the middle basin. Although the upper basin is near a protected area, and there are almost no anthropogenic activities, the presence of two positive samples could be due to river otters' territory overlapping with the middle basin,

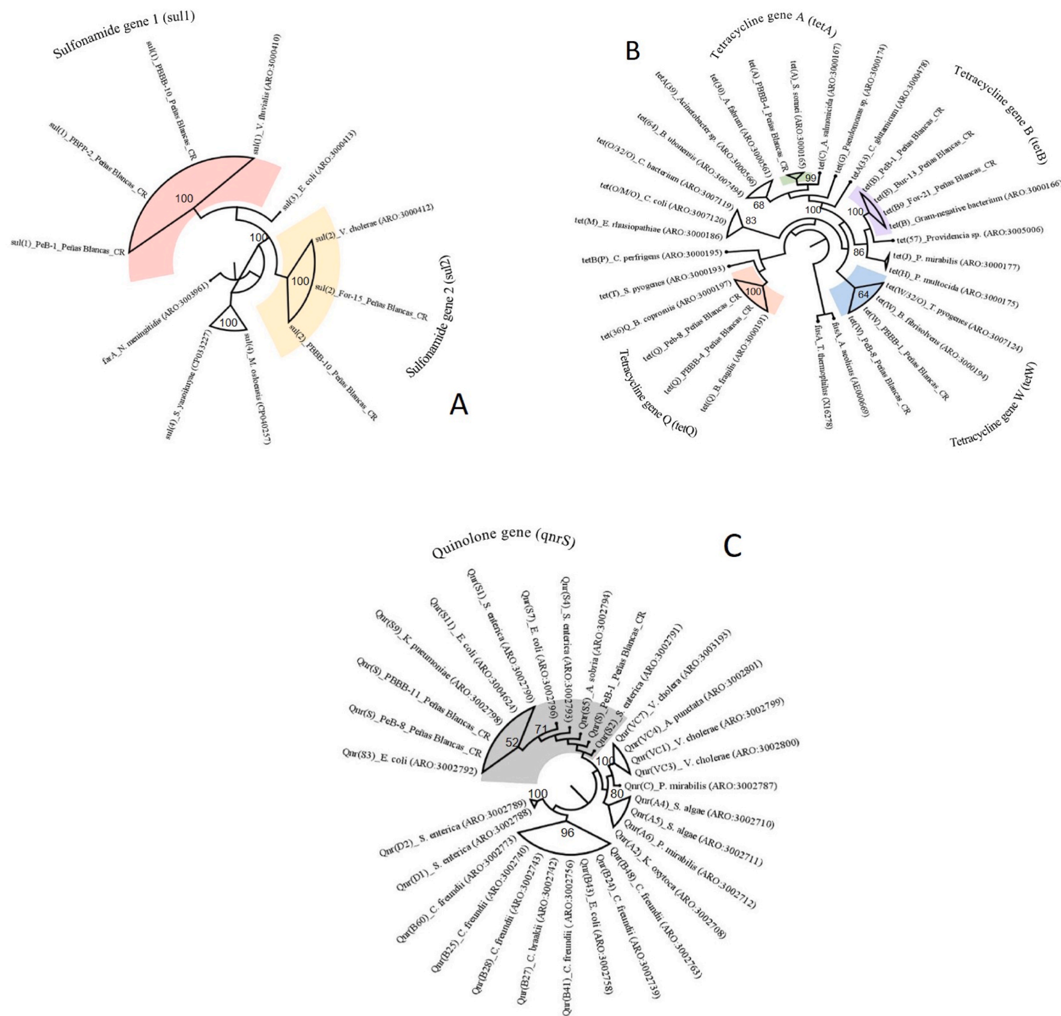

Fig. 3. Maximum likelihood-based phylogenetic analyses for validation of AMR gene families found in the otter's feces samples, inferred using IQ-TREE with model selection using ModelFinder and 1000 ultrafast bootstrap replicates (UFBoot), indicated as % in each node. AMR gene family phylogenetic tree figures are: A = Sulfonamides, B=Tetracyclines, and C = Quinolones. CARD (ARO) accession numbers of sequences used in the analysis are given in parentheses. Clusters between sequences from the database and those from Peñas Blancas-Costa Rica were colored: *tetA* = green, *sulI* = red, *sulII* = yellow, *qnrS* = grey, *tetW* = blue, *tetB* = purple and *tetQ* = orange.

as the study area was small and their home range size tends to be between 3 and 8 Km [57] depending on the season [58], forest proportion [33,59], and prey abundance [60]. As river otters can tolerate human presence and structures to a certain degree [61], those from the upper basin could contact streams contaminated with residues and/or contaminated prey when searching areas near human settlements and mobilizing ARGs to the protected area during their foraging time. Besides, waterborne bacteria in the river could also be resistant and could contaminate feces.

Within the middle basin, the finding of resistance genes for tetracyclines, sulfonamides, and quinolones could be attributed to their common use for agriculture, livestock, human medicine, and other anthropic activities [8,10,62,63]. Previous reports showed that the majority of genes found in farms, wastewater treatment plants, water, and soil were *tet* and *sul* [64], while *qrnS* has been commonly detected in aquatic environments polluted with fluoroquinolones [65], even if the water had a low concentration of antibiotics [66]. More importantly, previous studies have detected *tet* and *sul* genes within the skin microbiota of aquatic animals [67].

This study highlights the importance and usefulness of qPCR for ARG detection in wildlife to denote the impact of anthropic activities in natural environments [17,26,28,38]. The taxonomic placement analysis also confirmed its effectiveness in identifying specific ARGs, as shown in Fig. 3 ARGs clusters. Furthermore, all seven identified ARGs are associated with mobile genetic elements (MGEs): *qnrS*, *tetA*, *tetB*, *tetW*, *sulI*, and *sulII* are located on plasmids [68–70], *tetA, tetB*, and *tetQ* are associated with conjugative transposons, and *sulI* is additionally linked with the complex class 1 integron gene (*intI1*) [71,72].

MGEs can potentially colonize wildlife with ARGs with the aid of commensal and pathogenic bacteria [73,74]. As shown in a study where antibiotic resistance to beta-lactams (amoxicillin/clavulanate, ampicillin, penicillin), phenicols (chloramphenicol), macrolides

(clindamycin), aminoglycosides (gentamicin), quinolones (enrofloxacin), tetracyclines, and vancomycin were detected in *Enterococcus* spp. (a common commensal gut bacteria in river otters) [22]. And, *L. longicaudis* from the Peñas Blancas River gut bacteria includes bacteria phylum Actinobacteria, Bacteriodota, Firmicutes, and Proteobacteria [75], which are primary retainers and exchangers of ARGs mobile genes [76].

Although L. *longicaudis* from the Peñas Blancas River prefers to inhabit areas with high foliage (bushes, logs, and trees) and a wider river basin [33], the detection of ARGs in their feces demonstrates the influence of anthropic activities that surround the river basin and the potential for these anthropic disturbances to harbor, transfer, and potentially disrupt the normal gut microbiome of the otters, with resistance genes [14]. This situation has been observed in other Costa Rican wildlife species, such as jaguars (*Panthera onca*), pumas (*Puma concolor*) [17], and two-finger and three-finger sloths (*Choloepus hoffmanni* and *Bradypus variegatus*) [73]. The consequences for wildlife conservation in a country renowned for its biodiversity are currently unknown, but mitigation strategies are imperative to lessen the ARGs effects. In this sense, future studies regarding ARG detection in wildlife could benefit from our findings, as they serve as an example of the continuous need to investigate the still unknown and highly dynamic relationships among the environment, animals, and humans in the context of ARG dissemination.

4.1. Limitations

Although each sample was collected with care to avoid contamination from other environmental sources, several factors could have influenced our findings. The growth of antibiotic resistant bacteria (ARB) and the dynamics of ARG propagation and attenuation can be affected by the area's physical and chemical conditions, as well as antibiotic use in agriculture and livestock [8,77]. Moreover, land use in the Peñas Blancas River watershed is highly varied, encompassing not only agricultural and farming activities but also other anthropogenic processes. Runoff containing residues from these activities inevitably enters aquatic ecosystems in varying proportions, driven by diverse hydrological and mobilization processes that vary with regional seasonality [12,78]. But seasonality in ARGs occurrence and diversity is still poorly understood [28]. This raises the possibility that the collected fecal samples may have been contaminated by microorganisms carrying ARGs present in the environment. Nonetheless, it is important to note that wild life, including otters, inhabit this river and can be contaminated.

Fecal collection was preferred due to its non-invasive nature, eliminating the need to capture the animals. However, this method has the drawback of samples potentially not being fresh when obtained from a natural environment. Our fecal samples may have undergone abnormal bacterial proliferation or contamination from waterborne bacteria in the river. An additional limitation of our study was the variation in the number of samples collected across different areas (upper and middle basins), which can be attributed to the relative ease of locating feces during the dry season compared to the rainy season, when high river flows likely washed feces away from stones and other collection sites. Despite these limitations, qPCR detection phylogenetics analysis results, and the consistent identification of ARGs in our samples demonstrated a clear presence of these genes in the aquatic ecosystem of the Peñas Blancas River basin. This indicates that river otter populations are affected by the anthropogenic activities in the surrounding areas.

5. Conclusion

This pioneering study was able to denote that river otters inhabiting the Peñas Blancas watershed are accumulating ARGs and possibly disseminating them through the basin near protected areas (Bosque Eterno de los Niños) where no anthropic influence occurs. As ARGs dispersal, accumulation, and effects toward wildlife are still unknown, our study highlights the necessity of understanding the dynamic relationships among the environment, animals, and humans to better tackle the ever-growing AMR problem in both human and wildlife health.

The detection of three types of antibiotic resistance families (quinolones, tetracyclines, and sulfonamides) in otters' feces from the upper and middle basin, as well as multi-resistance samples, proves the impact of anthropogenic activities toward the natural environment. Future projects can benefit from our findings in campaigns centered on educating the public about the increasing risk of antimicrobial resistance in human and animal health, as they form part of the objectives of "One Health" and the "National Plan for Combating Antimicrobial Resistance 2018–2025 in Costa Rica: optimize the health of people, domestic animals, wildlife, and plants; through vigilance and control of AMR in our environment [5,79].

L. longicaudis is a top predator in their ecosystem. Detecting ARGs in otter feces might also indicate the presence of these genes in other species that inhabit the river. Prey such as crustaceans, fish, and other vertebrates could have a greater intake of ARGs, as most of them feed upon waste products from livestock or are in direct contact with wastewater from human settlements [22]. It is also important to acknowledge that the human-animal-environment interactions are too dynamic to confine the ARGs accumulation and dispersal to just wildlife. Therefore, we propose future studies to monitor all possible sources of ARG contamination in the river, and their presence in soil, water and river otters' preferred prey. Additionally, gaining a better understanding of the distribution and behavior of river otters within the Peñas Blancas River basin is necessary to secure the collection of fresher fecal samples for future ARG detection studies. A long-term study on the seasonal diversity of ARGs would also provide insights into gene dissemination influenced by weather conditions and mobilization processes. Such research could be complemented by surveys focused on antibiotic and pesticide use in the Peñas Blancas watershed.

CRediT authorship contribution statement

Aurora Guizado-Batista: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data

curation, Conceptualization. **Andrea Porres-Camacho:** Methodology, Investigation. **Seiling Vargas-Villalobos:** Writing – review & editing, Methodology, Investigation. **Manuel Cortez-Martínez:** Investigation. **Rodolfo Umaña-Castro:** Writing – review & editing, Validation, Software, Methodology, Investigation. **Carolina Sancho-Blanco:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Frank Solano-Campos:** Writing – review & editing, Software, Methodology, Investigation, Funding acquisition, Formal analysis. **Francisco Quesada-Alvarado:** Methodology, Investigation, Funding acquisition, Conceptualization. **Manuel Spínola-Parallada:** Writing – review & editing, Software, Methodology, Investigation, Data curation, Conceptualization. **Alexander Madrigal-Mora:** Methodology, Funding acquisition. **Adonay Jiménez-Serrano:** Methodology, Investigation. **Joshua Vargas-Calvo:** Methodology, Investigation, Formal analysis, Data curation. **Jenny Villalobos-Sequeira:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Kari Brossard Stoos:** Methodology. **Kinndle Blanco-Peña:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Data availability

Data will be made available on request.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Kinndle Blanco-Pena reports financial support was provided by International Training Program in Environmental Health over the Lifespan (D43TW011403, Claudio L & van Wendel de Joode B, PIs). Kinndle Blanco-Pena reports financial support was provided by Fondo Institucional de Desarrollo Académico, Universidad Nacional (FIDA). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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