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IDENTIFICACIÓN DE GENES DE RESISTENCIA ANTIMICROBIANA (ARGS) EN HECES DE NUTRIAS (*Lontra longicaudis*) Y PESTICIDAS EN AGUAS DEL RÍO PEÑAS BLANCAS, COSTA RICA

SUSTENTANTE

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Tesis presentada para optar al grado de Magister Scientiae en Medicina de la Conservación con énfasis en Salud Ecosistémica**. Cumple con los requisitos establecidos por el Sistema de Estudios de Posgrado de la Universidad Nacional, Heredia, Costa Rica.**

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VI. Resumen:

Dentro de la cuenca del río Peñas Blancas de Costa Rica, las poblaciones de nutria de río están en contacto con productos de desecho de actividades antropogénicas, tales como: ganadería, agricultura, acuicultura, asentamientos humanos, entre otras. Estas actividades dependen en gran medida del uso de antimicrobianos como promotores del crecimiento y tratamiento profiláctico en el ganado, así como del uso de diversos tipos de pesticidas para prevenir plagas agrícolas. El uso excesivo de estos químicos está relacionado con la formación de bacterias resistentes a los antibióticos (ARB), tanto en el tracto digestivo de los animales domésticos como en los suelos agrícolas. Cuando los productos de desecho contaminan los ecosistemas acuáticos, las nutrias de río pueden acumular ARB y genes de resistencia a los antibióticos (ARGs). La propagación de ARGs en el ecosistema es una gran preocupación en el campo de la conservación de la vida silvestre y de la salud humana, debido al potencial infeccioso que tienen estos microorganismos y lo difícil que resulta erradicarlos. La detección de ARGs en heces de *Lontra longicaudis* mediante qPCR representa una herramienta confiable para determinar el nivel de impacto que tienen las actividades antropogénicas sobre el ecosistema acuático del río Peñas Blancas. De manera similar, la detección de pesticidas en el agua de la cuenca de Peñas Blancas puede ayudar a comprender su potencial acción en el desarrollo del ARG. El monitoreo de pesticidas y ARGs en el medio ambiente y sus animales podría permitir una mejor comprensión de la dinámica humano-animal-ecosistema, para establecer mejores métodos de disposición de residuos y mitigar la resistencia a los antimicrobianos en entornos naturales.

Abstract:

Within the Peñas Blancas River basin of Costa Rica, river otter populations are in contact with waste products from anthropogenic activities: livestock, agriculture, aquaculture, human settlements, among others. These activities are strongly dependent on the use of antimicrobials as growth promoters and prophylactic treatment in livestock, as well as the use of various types of pesticides to prevent agricultural pests. The excessive use of antibiotics and pesticides is linked to the formation of antibiotic-resistant bacteria (ARB), both in the digestive tract of domestic animals and in agricultural soils. When waste products contaminate aquatic ecosystems, river otters can accumulate ARBs and antibiotic-resistance genes (ARGs). The spread of ARGs in the ecosystem is a great concern in the field of wildlife conservation and human health, due to the infectious potential that these microorganisms have and how difficult it is to eradicate them. The detection of ARGs in L. longicaudis feces by means of qPCR represents a reliable tool to determine the level of impact that anthropogenic activities have on the aquatic ecosystem of the Peñas Blancas River. Similarly, the detection of pesticides within the water of Peñas Blancas watershed can help understand their potential over ARG development. Pesticides and ARG monitoring within the environment and its animals could allow a better understanding of the dynamic human-animal-ecosystem, a so to establish better methods of waste disposal and mitigate antimicrobial resistance in natural environments.

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IX. Lista de abreviaturas:

AMR: Antimicrobial Resistance; Resistencia a los Antimicrobianos

ARB: Antibiotic Resistant Bacteria; Bacterias Antibiótico-Resistentes.

ARGs: Antibiotic Resistant Genes; Genes de Resistencia Antimicrobiana.

BeN: Children's Eternal Rainforest; Bosque Eterno de los Niños.

CARD: Comprehensive Antibiotic Resistance Database; Base de Datos Completa sobre Resistencia a los Antibióticos.

Ct: Threshold cycle; Ciclo umbral.

FIDA: Fondo Institucional de Desarrollo Académico, Universidad Nacional.

FRAC: Fungicide Resistance Action Committee; Comité de Acción para la Resistencia a Fungicidas.

Pr ICE: Costarrician Electrical Institute hydroelectric dam; Represa Hidroeléctrica del Instituto Costarricense de Electricidad.

HRAC: Herbicide Resistance Action Committee; Comité de Acción para la Resistencia a Herbicidas.

HGT: Horizontal gen transfer; Transferencia horizontal de genes.

IRAC: Insecticide Resistance Action Committee; Comité de Acción para la Resistencia a Insecticidas.

IRET: Instituto Regional de Estudios en Sustancias Tóxicas.

LAGen: Laboratorio de Análisis Genómico.

Lu Pk: La Lucha settelment; Poblado La Lucha

MGE: mobile genetic elements; Elementos genéticos móviles.

ML: Maximum likelihood; Máxima verosimilitud.

MRD: Multidrug-Resistant; Resistencia a Múltiples Drogas.

PBb: Burro Brujo river; río Burro Brujo.

PCR: Polymerase Chain Reaction; Reacción de Cadena Polimerasa

qPCR: Real-time Polymerase Chain Reaction; Reacción de Cadena Polimerasa en Tiempo Real.

WHO: World Health Organization; Organización Mundial de la Salud.

X. Descriptores:

Genes de resistencia a antibióticos

Ecosistema acuático

Nutrias de río

Actividades antropogénicas

Microbioma multi-resistente

Pesticidas

XI. Introducción general:

La resistencia antimicrobiana (AMR) es un fenómeno de adaptación que permite a los microorganismos (bacterias, virus, hongos y parásitos) desarrollar mecanismos fisiológicos contra los compuestos antimicrobianos que se utilizan para matarlos o controlar su proliferación (O'Neill, 2016). La capacidad de las bacterias en adquirir resistencia antimicrobiana ha sido el foco de numerosos estudios en los últimos 30 años (Huemer et al., 2020). En ese sentido, se ha encontrado que han desarrollado un amplio rango y diversidad de resistencias ante fármacos, pesticidas u otras sustancias químicas sintetizadas por el ser humano, convirtiéndose en una de las mayores amenazas contra la salud mundial (Llor & Bjerrum, 2014).

En bacterias, la AMR puede ser mediada por genes de resistencia antimicrobiana (ARGs) (Hu et al., 2017). Los ARGs tienen diversas formas de adquirirse y desarrollar AMR, tales como: interacción con antimicrobianos que se producen naturalmente (resistencia intrínseca), mutación y transferencia horizontal de genes (HGT) (resistencia adquirida) y resistencia transitoria al interactuar con estresores específicos en su ambiente (resistencia adaptativa) (Christaki et al., 2020).

Las actividades humanas juegan un papel importante en el incremento del desarrollo y esparcimiento de los ARGs (WHO, 2021). A nivel mundial, existe una dependencia hacia los fármacos para tratar enfermedades e infecciones en humanos y animales domésticos, pero se utilizan incluso donde no son necesarios (Dadgostar, 2019). Esto mismo aplica para los pesticidas, que son utilizados para combatir plagas y aumentar la productividad de los cultivos (Bernardes et al., 2015; Edwards, 1993).

Dentro de la industria agropecuaria, los fármacos de uso veterinario (por ejemplo, penicilinas, quinolonas, tetraciclinas, sulfonamidas, entre otros) son utilizados como promotores de crecimiento y profilaxis (Berglund, 2015; Zalewska et al., 2021). El tracto digestivo y los desechos de estos animales cargados de antibióticos convierten a las áreas aledañas a granjas, corrales, graneros o

pasturas en fuentes de contaminación de ARB y ARGs al medio ambiente (Amarasiri et al., 2020; Odonkor & Addo, 2011; Singer et al., 2016; Zalewska et al., 2021). Por su parte, los pesticidas tienen la capacidad de promover el desarrollo de resistencia cruzada o co-resistencia ante distintos compuestos activos no relacionados, formando resistencia a múltiples drogas (MRD), además de intensificar la adquisición de ARGs por medio de HGT en la conjugación entre bacterias (Curutiu et al., 2017; Qiu et al., 2022). Ambas actividades normalmente se desarrollan cerca unas de otras, amplificando la capacidad de las comunidades densas de bacterias que se encuentran ahí para seleccionar, mantener, transferir y diseminar sus ARGs (Christaki et al., 2020; Singer et al., 2016).

Todos estos productos (antibióticos, pesticidas, ARB y ARGs) terminan en los sistemas de desechos y contaminan el ecosistema en proporciones distintas, siendo los sistemas acuáticos los primeros aceptores (Amarasiri et al., 2020; Qiu et al., 2022). Los efectos relacionados con la introducción de ARGs y ARB en ambientes naturales y sus animales, así como su prevalencia a lo largo de la red trófica, siguen siendo poco conocidos (Singer et al., 2016). Se sabe que la contaminación por ARGs y ARB conlleva a la disminución de las zonas de amortiguamiento e incremento del resistoma de los microorganismos (ARGs en comunidades de bacterias patógenas y no patógenas) que se desarrollan en estas áreas (McEwen & Collignon, 2018). Y la colonización de ARGs en la microbiota natural de los animales se ha relacionado con el desbalance del sistema inmune, lo que favorece la susceptibilidad a infecciones por cambios en el metabolismo (Yoo et al., 2020) y las alteraciones en su comportamiento (por ejemplo, el desarrollo de depresión) (Yang et al., 2020).

Los animales silvestres pueden convertirse en bioindicadores de la presencia y dispersión de ARGs, siendo una herramienta importante para determinar el nivel de impacto que poseen las actividades antropogénicas sobre los ambientes naturales (Cevidanes et al., 2020; Dias et al., 2022; Fernandes et al., 2022). En este sentido, las nutrias de río (*Lontra longicaudis*) son un buen candidato para la evaluación de la diseminación de ARGs en ecosistemas acuáticos.

Estos mustélidos son depredadores de alto nivel en los ríos, alimentándose de una amplia variedad de presas: crustáceos, peses y pequeños vertebrados (Navarro-Picado et al., 2017). Las poblaciones de nutrias distribuidas a lo largo de la cuenca hidrográfica del río Peñas Blancas, Costa Rica, se encuentran en contacto directo e indirecto con las actividades antropogénicas aledañas. Estos individuos podrían estar acumulando ARGs al consumir presas o entrar en contacto con aguas contaminadas por las actividades humanas cercanas a los ríos (Semedo-Lemsaddek et al., 2018). El uso de sus heces para la detección de dichos genes es un método no invasivo de recolección de muestras y la implementación de qPCR permite detectar genes específicos que confieren resistencia antimicrobiana, facilitando el proceso de monitoreo de estos contaminantes emergentes en el ecosistema (Angulo et al., 2023; Nieto-Claudin et al., 2019; Sacristán et al., 2020; Vargas-Villalobos et al., 2024).

Mediante la presente investigación se desarrollaron dos artículos científicos. El primero se encuentra enfocado en la detección de ARGs en muestras de heces de nutrias recolectadas entre 2019 y 2022, con el propósito de establecer la presencia, diversidad y distribución de estos genes a lo largo de la cuenca del Peñas Blancas. Igualmente, se buscó determinar el posicionamiento taxonómico de los ARGs detectados, con la finalidad de asegurar la validez y confiabilidad de la detección de estos genes mediante qPCR, así como su relación con prominentes filos de bacterias que son albergadores y transmisores de ARGs.

En el segundo artículo, se analizaron muestras de heces de nutria y agua recolectadas durante el 2022 en la cuenca del río Peñas Blancas. El propósito de este artículo consistía en determinar la relación de la presencia de pesticidas en el agua y los ARGs dentro de las heces de nutrias, así como la distribución de ambos a lo largo de la cuenca.

Ambos artículos buscan ampliar el nivel de entendimiento del vínculo humano-animal-ecosistema, para brindar información sobre el impacto de las actividades antropogénicas en los sistemas acuáticos. Los datos que se generen podrán ser utilizados por los tomadores de decisiones para la mitigación de la presencia de estos compuestos en la cuenca del río Peñas Blancas.

XII. Conclusiones generales:

Este estudio pionero determinó que las poblaciones de nutrias del río Peñas Blancas se encuentran acumulando ARGs relacionados a familias de antibióticos comúnmente utilizados en la industria agropecuaria y tratamiento humano. Estos genes se agruparon en las siguientes familias de antibióticos: tetraciclinas (*tetA, tetB, tetQ,* y *tetW*), quinolonas (*qrnS*) y sulfonamidas (*sulI* y *sulII*). No se detectaron genes otorgan resistencia a las familias: beta-lactámicos (***bla*$_{\text{CTX-M}}$**), diaminopirimidinas (*dfraA12*), fenicoles (*catII*) y macrólidos (*ermB*).

La mayoría de las muestras de heces presentaban gran diversidad de ARGs en las áreas más antropogenizadas de la cuenca (parte media), con 3 muestras donde se detectó un microbioma multirresistente (una muestra fecal que presenta al menos tres ARGs que codifican resistencia a diferentes grupos de antimicrobianos). Sin embargo, dentro de la parte alta se detectó una muestra con cuatro ARGs (*tetA, tetB, sulI,* y *sulII*). Esto resulta preocupante, debido es una zona cercana al Bosque Eterno de los Niños (BeN).

Tomando en consideración los resultados anteriormente mencionados, se podría especular que las nutrias de río podrían estar acumulando estos genes al entrar en contacto con fuentes de agua contaminada cercanas a las actividades antropogénicas de la parte media de la cuenca. Esto podría resultar en su dispersión en sitios prístinos y en el aumento de los riesgos de la salud, tanto humana como de la vida silvestre.

La cuenca del río Peñas Blancas presenta también una contaminación significante de pesticidas relacionados con actividades humanas, tales como las agrícolas y ganaderas (por la presencia de pastos). Por ejemplo, se determinó la presencia de trazas de pesticidas de uso restringido en Costa Rica (clorpirifos y etoprofos) en la parte alta de la cuenca, así como de otros que han sido relacionados con la propagación y mantención de resistencia antimicrobiana (por ejemplo, clorpirifos, diazinon,

diuron, imidacloprid, y terbutrin). Por tanto, nuestros resultados resaltan el impacto de las prácticas agrícolas que se desarrollan a lo largo de la cuenca del Peñas Blancas en la salud del ecosistema, así como su posible aporte a la diseminación de ARGs en la vida silvestre. La presencia de estos pesticidas también denota los posibles riesgos que su uso irresponsable representa para el ecosistema, debido a que estos compuestos tienden a ser muy tóxicos (especialmente en especies acuáticas) y son capaces de persistir en los suelos y agua por semanas o años.

Este estudio demostró la contaminación del ecosistema acuático con ARGs y plaguicidas a causa de actividades antropogénicas. Con base en estos resultados, generó información valiosa sobre sus posibles repercusiones en la salud ambiental, tomando como base la presencia de los contaminantes antes mencionados en un bioindicador de alto nivel trófico y en su hábitat.

XIII. Recomendaciones generales:

Se debe tener en cuenta el comportamiento ecológico de la nutria de río con la finalidad de obtener muestras de heces con mejor calidad para su análisis. Para ello, se requiere realizar más estudios sobre sus hábitats de preferencia, lo que permitiría un mejor entendimiento de su distribución en la cuenca. Muestras de heces de mayor calidad permitirían una detección de ARGs con mayor confiabilidad, sin tener que recurrir a análisis de secuenciación genética para determinar la validez de aquellas que denotaron niveles de ciclo umbral (Ct) de más de 30.

Se recomienda aumentar el número de ARGs que se determinen en las muestras de heces. Si bien es necesario detectar aquellos genes que son comúnmente asociados a los antibióticos más utilizados en actividades antropogénicas, se podrían estar ignorando otros que también representan un peligro para la salud ambiental.

Se espera que nuestros resultados sean utilizados en campañas de capacitación relacionadas con la educación sobre los riesgos de la resistencia antimicrobiana, así como para la regulación del uso de pesticidas y sus efectos ante la salud humana y la vida silvestre.

Futuros estudios dentro de la cuenca del Peñas Blancas podrían enfocarse en detectar ARGs en las presas de las nutrias y agua de los ríos, con el fin de aumentar nuestro conocimiento sobre la compleja interacción humano-animal-ambiente.

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XV. Artículo 1: Antimicrobial-resistant genes in feces from otters (*Lontra longicaudis*) within the Peñas Blancas river basin, Costa Rica

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Heliyon

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

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ABSTRACT

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 within 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. Subsequent Maximon-likelihood-base taxonomic placement was conducted using sequences from 16 ARG-positive samples. 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". Seven distinct ARGs were identified: *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 revealed taxonomic association of all detected ARGs, which were compared with The Comprehensive Antibiotic Resistance Database. Furthermore, these ARGs were strongly associated with prominent bacterial phyla known for harboring and transferring resistance genes, including Actinobacteria, Bacteroidota, Firmicutes, and Proteobacteria. These phyla have been identified in river otters gut microbiota, suggesting a potential colonization of microbes harboring ARGs. These findings underscore the importance of comprehending the spread of microbes bearing 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.

KEYWORDS: Anthropogenic activities, antimicrobial resistance, aquatic ecosystems, mobile genetic elements, multi-resistance microbiome, phylogeny, sentinels, wildlife.

FUNDING STATEMENT

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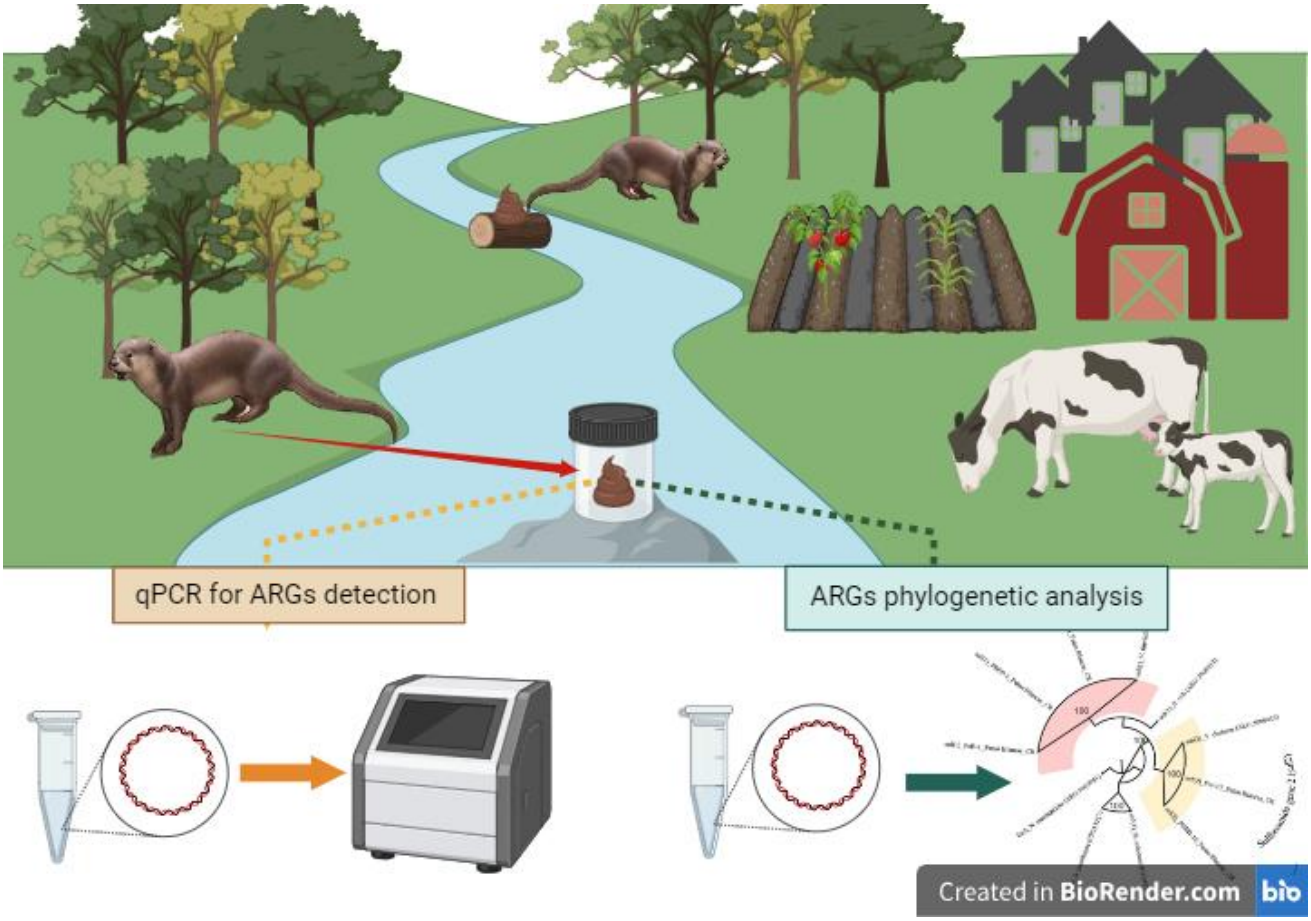
HIGHLIGHTS

- ARGs found in river otters feces collected in a Costa Rican protected area (Bosque Eterno de los Niños).
- Multi-resistance microbiome found in otter feces samples.
- Linkage between ARGs sample sequences with prominent bacteria phyla that can harbor and exchange them.
- Risk of ARG colonization and alteration of normal river otters gut microbiota.

GRAPHICAL ABSTRACT



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 (O'Neill, 2016). Within bacteria, antibiotic resistance is mediated by antibiotic-resistant genes (ARGs) (Hu et al., 2017). 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) (Christaki et al., 2020). The capture, accumulation, and dissemination of ARGs are highly mediated by mobile genetic elements (MGE), such as plasmids, transposons, integrons, integrative conjugative elements, and genomic islands, which play a crucial role in HGT (Partridge et al., 2018).

AMR is a natural adaptation phenomenon, but human activities have an increasing impact on the development and spreading of ARGs (McEwen & Collignon, 2018; WHO, 2019; 2021). For instance, livestock is a significant source of environmental ARGs (He et al., 2020), receiving four times the amount of drugs and antibiotics by humans for growing and prophylaxis treatment (Vanegas-Múnera & Jiménez-Quiceno, 2020), such as penicillin, quinolones, tetracyclines, sulfonamides, and others (Zalewska et al., 2021). The increase in pesticide use can be attributed to pests developing broader tolerance, promoting acquired and adaptive resistance in bacteria. This phenomenon is exacerbated by pesticides sharing similar chemical components with antibiotics, such as tetracyclines and streptomycin (Curutiu et al., 2017). It is known that 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 (Amarasiri et al., 2020; Christaki et al., 2020; Curutiu et al.,

2017; Singer et al., 2016; Zalewska et al., 2021). Therefore, aquatic environments have been proposed as predominant ARGs reservoirs and transmission routes as they are easily accessible for anthropogenic activities (Amarasiri et al., 2020; Hu et al., 2017).

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 (Dolejska & Literak, 2019; Kraemer et al., 2019; Plaza-Rodríguez et al., 2021). 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 (Angulo et al., 2023; Blanco-Peña et al., 2017, 2024; Vargas-Villalobos et al., 2024).

River otters (*Lontra longicaudis*) have been employed as bioindicators to assess the occurrence and dispersion of ARGs in aquatic environments, primarily attributed to their exposure to contaminated water sources and prey (Dias et al., 2019; Semedo-Lemsaddek et al., 2018). These mustelids exhibit varying degrees of interaction with diverse anthropogenic activities occurring throughout the Peñas Blancas River basin (Inder, 2015, 2016). 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 (Angulo et al., 2023; Blanco-Peña et al., 2024; Nnadozie & Odume, 2019; Sacristán et al., 2020; Vargas-Villalobos et al., 2024). Also, a phylogenetic analysis of these ARG sequences would help

pinpoint the phylum of bacteria with considerable potential to transfer these genes, as mobile ARGs transmission is mainly controlled by bacterial phylogeny (Hu et al., 2016). Thus, if a river otter's environment is frequently exposed to antibiotics and has a phylogenetic predisposition, ARGs could alter the antibiotic resistome from the bacterial communities of their gut microbiome and natural environments (Hu et al., 2017).

This pioneering study used qPCR and phylogenetic analysis to assess the presence of ARGs within *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 antimicrobial resistance (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"(SINAC, 2016). Its forest coverage includes premontane humid forests, with temperatures between 18 and 24°C year-round (Holdridge, 1967; Tosi, 2011). 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 (Inder, 2015, 2016; Reyes et al., 2001).

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 (Navarro-Picado et al., 2017). 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. Negative controls with distilled water were carried out to identify possible 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′-

CCTGAAGTAAGAACCAGATG-3′) (Cassens et al., 2000; Pérez-Haro et al., 2005; Valqui, 2010).

The 16s rRNA gene, an essential taxonomic marker for bacterial communities (Villalpando et al., 2018), 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. Table 1 shows the 12 ARGs selected for this study, considering the most prominent antimicrobial families used in anthropogenic activities (Curutiu et al., 2017; He et al., 2020; Zalewska et al., 2021). A sample was considered ARG positive if the Ct was less than 30, but if it was close to it, it was sequenced to clarify whether it was positive. The percentage charge of an ARG was calculated through the formula:

$$\log10(\mathrm{ARG\%}) = 2 + 0{,}33 * (\mathrm{Ct16s} - \mathrm{CtgenARG})$$

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 (Angulo et al., 2023, 2023; Nieto-Claudin et al., 2019; Sacristán et al., 2020). The results were expressed in log10 of the hypothetical percentage of bacteria each gene presented for the ARGs percentage load. All qPCRs were done in a CFX96 Touch Real-Time PCR Detection System, using SYBR Green® (Jiang et al., 2013).

PCR conditions were optimized per primer pair in the MacVector software. Primer pairs are in Anexo 2. Controls for *dfrA12*, *catII*, *erm(B)*, *qnrS*, *tetB*, *tetQ*, *tetW*, and *tetY* were produced 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*$_{\text{CTX-M,}}$ *sulI*, *sulII*, and *tetA.*

Table 1. Mechanism of resistance, reference and antibiotic-resistant genes selected for river otter feces analysis within 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*$_{\text{CTX-M}}$**	Hydrolysis	CTATGGCACCACCAACGATA ACGGCTTTCTGCCTTAGGTT	(Marti et al., 2013)
Diaminopyrimidines	***dfrA12***	Target protection enzymes	GAGCTGAGATATACACTCTGGCACT GAGCTGAGATATACACTCTGGCACT	(Grape et al., 2007)
Phenicols	***catII***	Inactivating enzymes	GATTGACCTGAATACCTGGAA CCATCACATACTGCATGATG	(Yoo et al., 2003)
Macrolides	***erm(*B)**	Ribosomal methylation	CCGAACACTAGGGTTGCTC ATCTGGAACATCTGTGGTATG	(Di Cesare et al., 2013)
Quinolones	***qnrS***	Target protection enzymes	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	(Marti & Balcázar, 2013)
Sulfonamides	***sulI***	Ribosomal protection	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	(Luo et al., 2010)
	sulII	Ribosomal protection	TCCGGTGGAGGCCGGTATCTGG CGGGAATGCCATCTGCCTTGAG	(Luo et al., 2010)

Antibiotic Family	Gene	Mechanism of resistance	Forward and Reverse Primers	Reference
Tetracyclines	***tetA***	Efflux pump	TCAATTTCCTGACGGGCTG GAAGCGAGCGGGTTGAGAG	(Keenum et al., 2022)
	tetB	Efflux pump	CGCCCAGTGCTGTTGTTGTC CGCGTTGAGAAGCTGAGGTG	(Goswami et al., 2008)
	tetQ	Ribosomal protection	AGAATCTGCTGTTTGCCAGTG CGGAGTGTCAATGATATTGCA	(Aminov et al., 2001)
	tetW	Ribosomal protection	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	(Aminov et al., 2001)
	tetY	Efflux pump	ATTTGTACCGGCAGAGCAAAC GGCGCTGCCGCCATTATGC	(Aminov et al., 2001)

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) (Anexo1).

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.Taxonomic placement analysis:

Sanger sequencing of ARGs PCR products from 16 ARG-positive samples was carried out (Macrogen Inc., Korea). Samples for each confirmed ARG were selected according to their Ct values (<25). The ARG sequences were used as queries to search sequences on the Comprehensive Antibiotic Resistance Database, CARD v3.2.9 (Alcock et al., 2023) using BLASTn in Geneious Prime 2019.2.3 to obtain homologous sequences. Sequences resulting from these searches that had more than 65% identity and 60% coverage were considered homologous. Then, datasets were aligned using MUSCLE v5 (Edgar, 2022), and the removal of poorly aligned regions was carried out with TrimAl v1.4 (Capella-Gutiérrez et al., 2009). Maximum likelihood (ML) phylogenetic trees were inferred with IQ-TREE v2.3 (Nguyen et al., 2015), employing ModelFinder (Kalyaanamoorthy et al., 2017) 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 (Hoang et al., 2017; Minh et al., 2013) 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 (Figure 1).

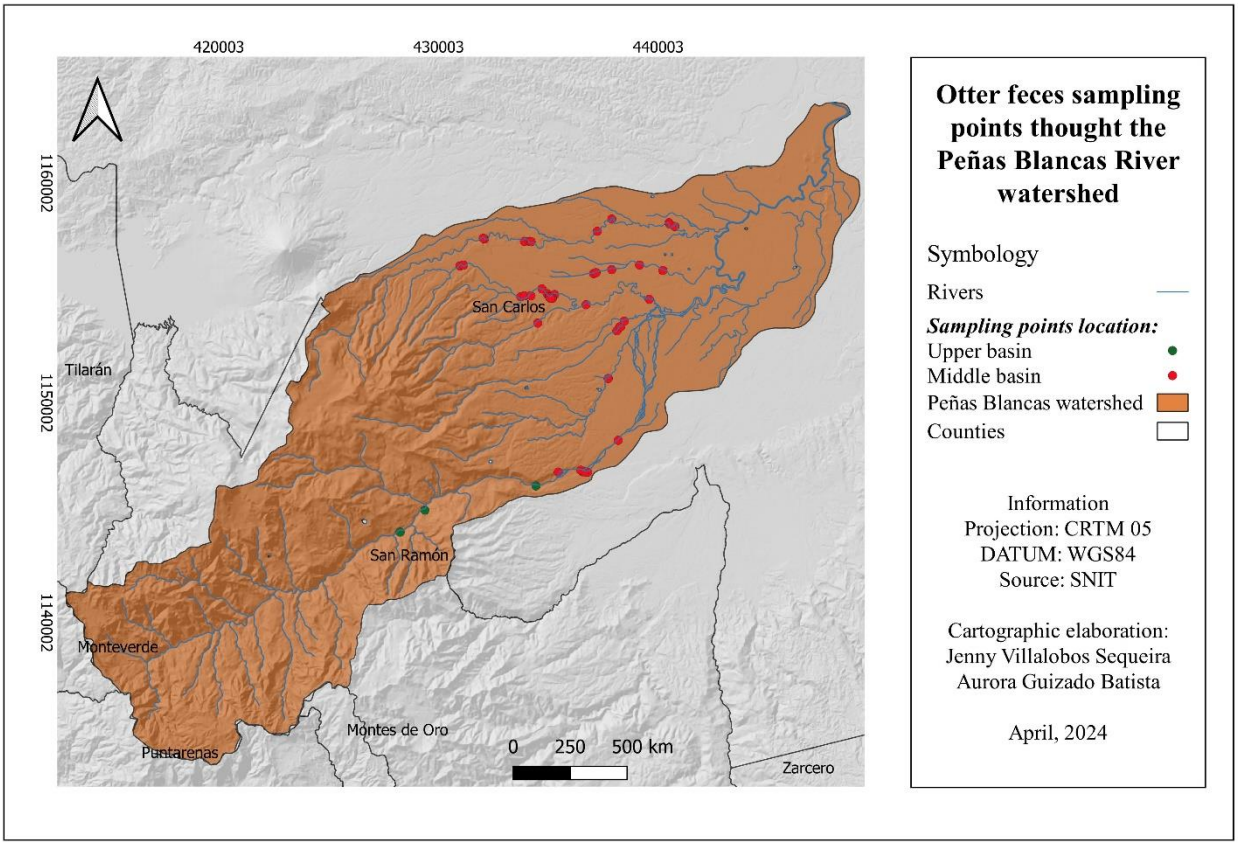


Figure 1. Sampling points with river otter feces positive for 16s rRNA qPCR through Peñas Blancas River watershed. **2**

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.

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

The correlation analysis revealed significant inter-family relationships within the data. For instance, *tetB* exhibited a notably stronger positive correlation with *sulI*, while *sulII* displayed correlations with *tetA*, *qnrS*, *tetW*, and *sulI* (Figure 2). Nevertheless, in contrast to findings from other studies conducted in the country, these results appear less consistent, suggesting a prior notion that the presence of ARGs varies based on regional disparities in human activities and substance usage (Blanco-Pena et al., 2017; Vargas-Villalobos et al., 2023; Blanco-Pena et al., 2024).

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 ARG-positive fecal samples can be viewed in Anexo3.

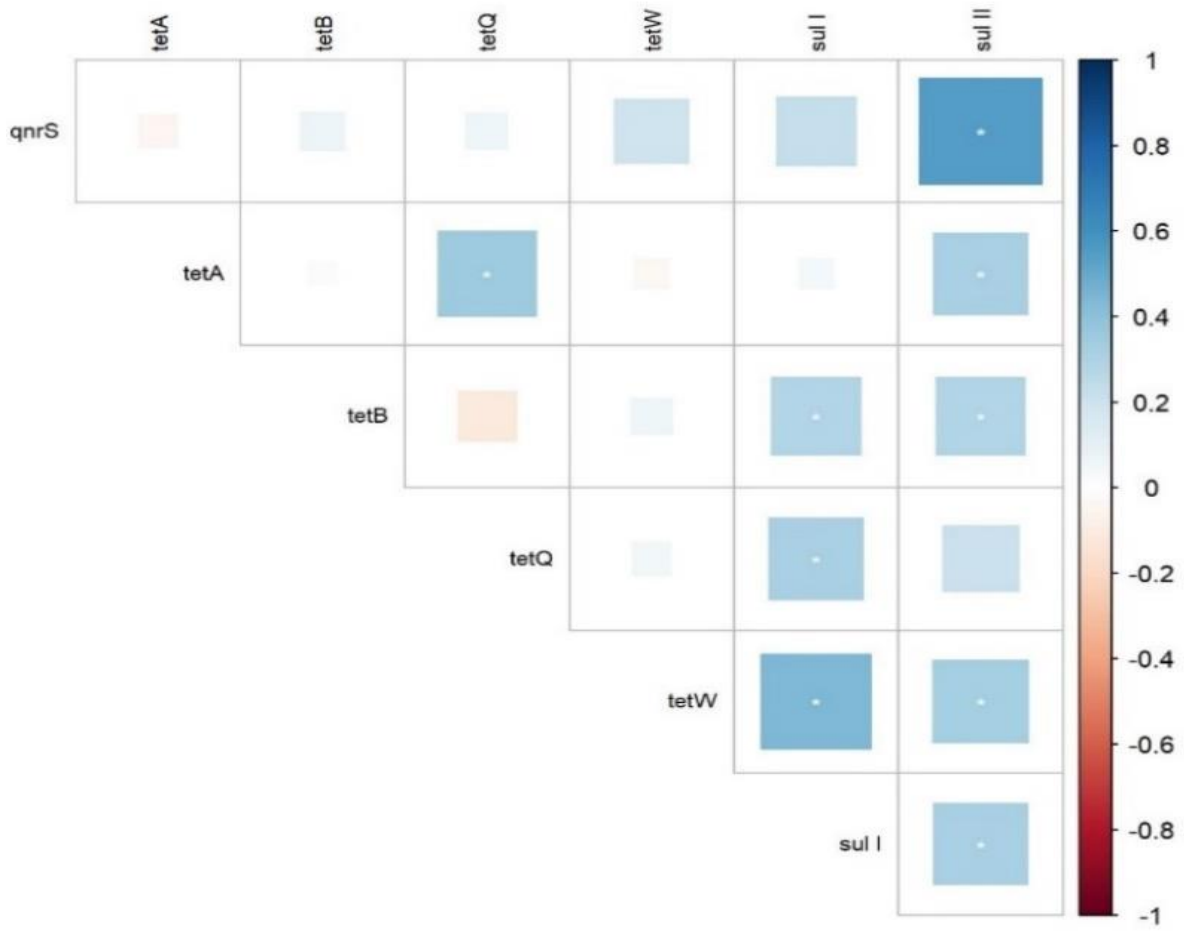
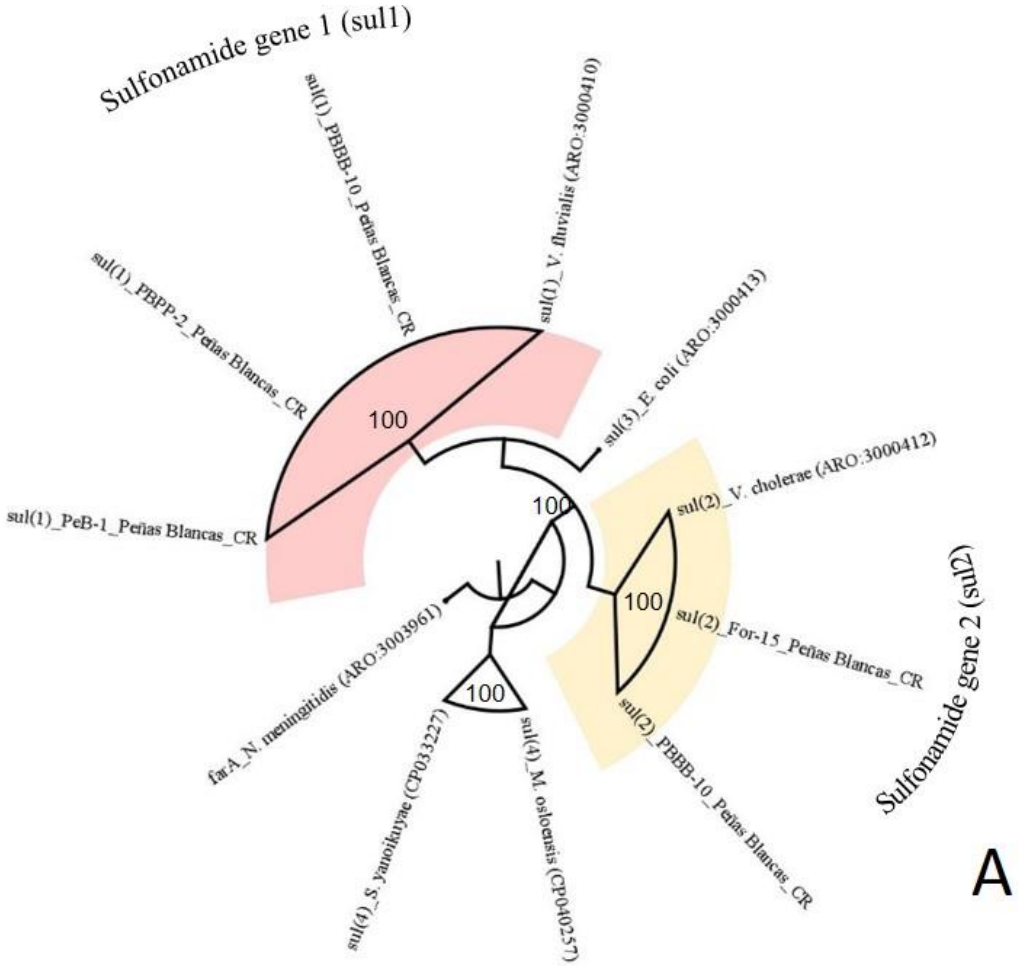
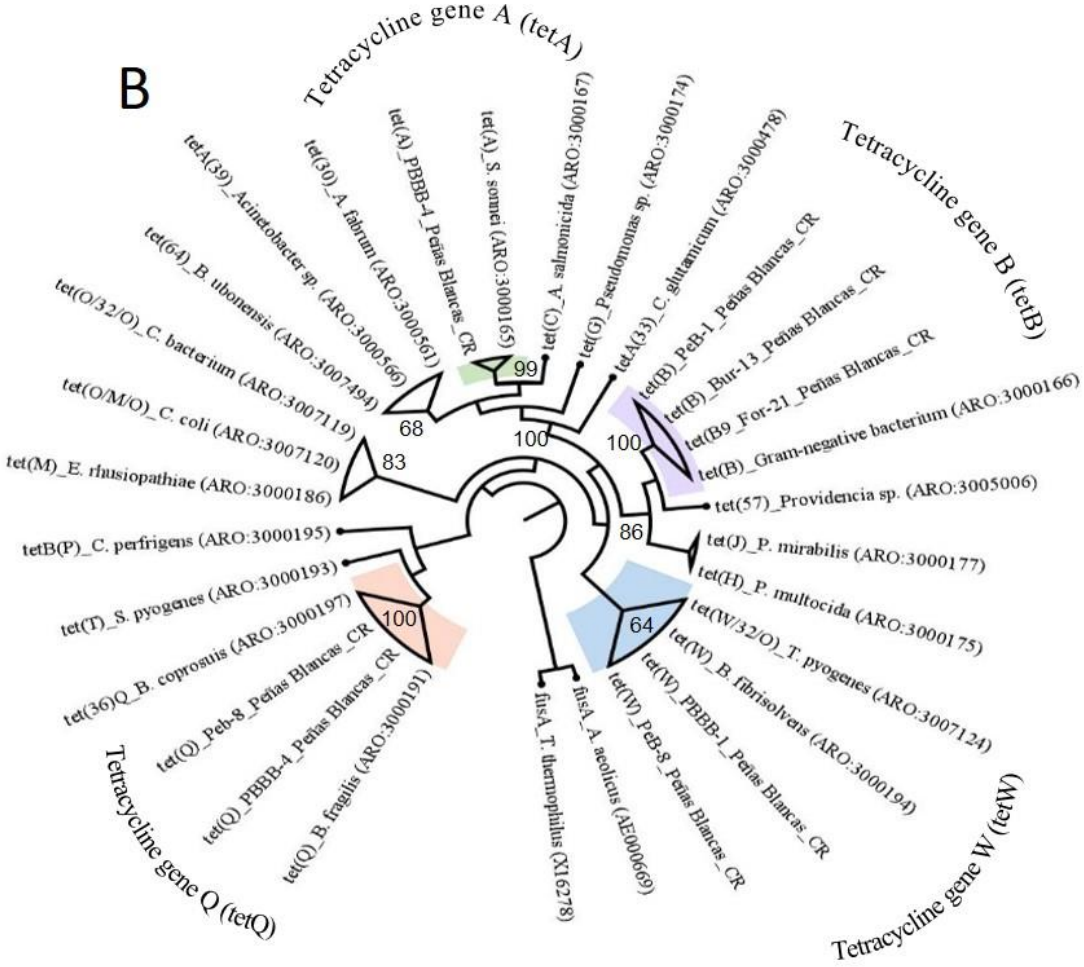


Figure 2. Correlation among resistance genes identified in River Peñas Blancas basin otter feces. The square size indicates the strength of the relationship between variables, the color signifies whether the relationship is positive or negative, and the asterisk indicates significance.


Sulfonamide gene 1 (sul1)
sul(1)_PBBB-10_Peñas Blancas_CR
sul(1)_V. fluvialis (ARO:3000410)
sul(1)_PBPP-2_Peñas Blancas_CR
sul(3)_E. coli (ARO:3000413)
100
sul(1)_PeB-1_Peñas Blancas_CR
100
sul(2)_V. cholerae (ARO:3000412)
100
sul(2)_For-15_Peñas Blancas_CR
farA_N. meningitidis (ARO:3003961)
100
sul(4)_S. yanoikuyae (CP033227)
sul(4)_M. oslonensis (CP040257)
sul(2)_PBBB-10_Peñas Blancas_CR
Sulfonamide gene 2 (sul2)
A


B
Tetracycline gene A (tetA)
Tetracycline gene B (tetB)
Tetracycline gene Q (tetQ)
Tetracycline gene W (tetW)
tet(A)_PBBB-4_Peñas Blancas_CR
tet(A)_S. sonnei (ARO:3000165)
tet(C)_A. salmonicida (ARO:3000167)
tet(G)_Pseudomonas sp. (ARO:3000174)
tetA(33)_C. glutamicum (ARO:3000478)
tet(30)_A fabrum (ARO:3000561)
tetA(39)_Acinetobacter sp. (ARO:3000566)
tet(64)_B. ubonensis (ARO:3007494)
tet(O/32/O)_C. bacterium (ARO:3007119)
tet(O/M/O)_C. coli (ARO:3007120)
tet(M)_E. rhusiopathiae (ARO:3000186)
tetB(P)_C. perfrigens (ARO:3000195)
tet(T)_S. pyogenes (ARO:3000193)
tet(36)Q_B. coprosuis (ARO:3000197)
tet(Q)_Peb-8_Peñas Blancas_CR
tet(Q)_PBBB-4_Peñas Blancas_CR
tet(Q)_B. fragilis (ARO:3000191)
fusA_T. thermophilus (X16278)
fusA_A. aeolicus (AE000669)
tet(W)_PeB-8_Peñas Blancas_CR
tet(W)_PBBB-1_Peñas Blancas_CR
tet(W/32/O)_T. pyogenes (ARO:3007124)
tet(W)_B. fibrisolvens (ARO:3000194)
tet(J)_P. mirabilis (ARO:3000177)
tet(H)_P. multocida (ARO:3000175)
tet(57)_Providencia sp. (ARO:3005006)
tet(B)_PeB-1_Peñas Blancas_CR
tet(B)_Bur-13_Peñas Blancas_CR
tet(B9_For-21_Peñas Blancas_CR
tet(B)_Gram-negative bacterium (ARO:3000166)
99
68
83
100
100
100
86
64
100

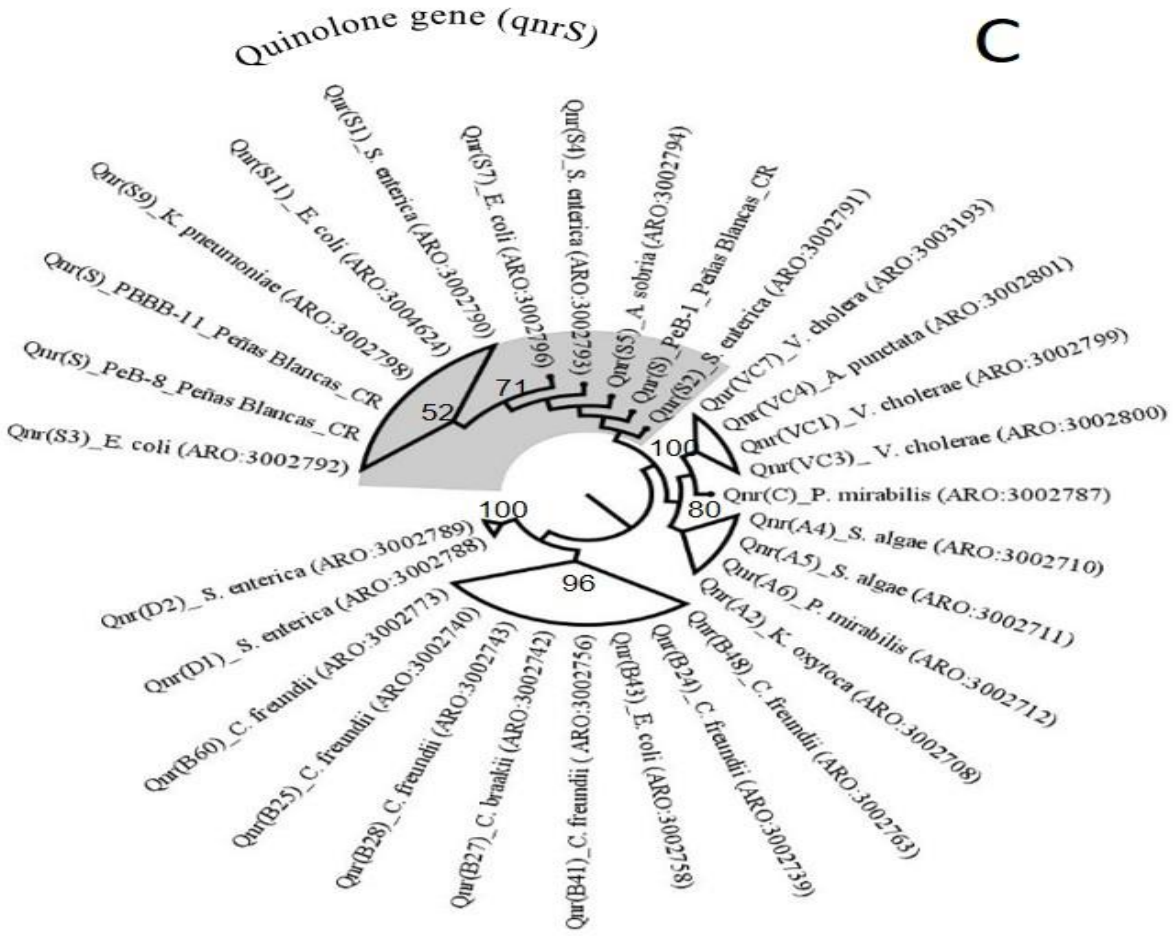


Figure 3. Maximum likelihood-based taxonomic placement topology for molecular validation of the ARGs found in the otter's feces samples, inferred using IQ-TREE with model selection using ModelFinde and 1000 ultrafast bootstrap replicates (UFBoot), indicated as % in each node. AMR gene family phylogenetic 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.

4. DISCUSSION

Throughout the Peñas Blancas River, seven genes responsible for resistance towards 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 (Causanilles et al., 2017; Spongberg et al., 2011). We did not find *bla*$_{CTX}$ (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 forms part of 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, as the study area was small and their home range size tends to be between 3 to 8 Km (Gallo-Reynoso, 1989) depending on the season (Ortega et al., 2012), forest proportion (Navarro-Picado et al., 2017; Smith et al., 2020), and prey abundance (Rheingantz et al., 2012). As river otters can tolerate human presence and structures to a certain degree (Kruuk, 2006), 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 contaminate feces.

Within the middle basin, the finding of resistance genes for tetracyclines, sulfonamides, and quinolones could be attributed to their common usage for agriculture, livestock, human medicine, and other anthropic activities (Gutiérrez et al., 2010; He et al., 2020; Pikkemaat, et al., 2016; Zalewska et al., 2021). Previous reports showed that the majority of genes found within farms, wastewater treatment plants, water, and soil were *tet* and *sul* (Zhuang et al., 2021). While *qrnS* has been commonly detected in aquatic environments polluted with fluoroquinolones (Adachi et al., 2013), even if the water had a low concentration of antibiotics (Castrignanò et al., 2020). More importantly, previous studies have detected *tet* and *sul* genes within the skin microbiota of aquatic animals (Hong et al., 2018).

Another point that must be established is that the variation in the number of samples collected across different areas (upper and middle basins) may be attributed to the ease of locating feces during the dry season compared to the rainy season. During high flows, feces were more likely to be washed away from stones.

This study highlights the importance and usefulness of qPCR for ARG detection in wildlife to denote the impact of anthropic activities in natural environments (Angulo et al., 2023; Cevidanes et al., 2020; Nieto-Claudin et al., 2019; Sacristán et al., 2020). The phylogenetic analysis also confirmed its effectiveness in identifying specific ARGs, as shown in Figure 3 ARGs clusters. Additionally, *tetA*, *tetB*, *qrnS*, *sulI* and *sulII* clusters grouped sequences that perfectly matched bacteria species within phylum Proteobacteria, *tetQ* for Bacteriodota, and *tetW* in Actinobacteria and Firmicutes. Yet, the CARD data base results revealed several other bacteria species with perfect matches resistomes with our ARG-positive samples. These species included the following phyla: Actinobacteria (*tetA*, *tetW*, and *sulI*), Bacteroidota (*tetA*

and *sulII*), Campylobacterota (*tetA*), Deinococcota (*tetA*), Firmicutes (*tetA, tetQ* and *sulI*), and Fusobacterium (*tetA* and *tetQ*). This finding is important considering that Actinobacteria, Bacteriodota, Firmicutes, and Proteobacteria are ARGs mobile genes' primary retainers and exchangers (Hu et al., 2016).

All seven identified ARGs are associated with mobile genetic elements (MGEs). Specifically, *qnrS*, *tetA*, *tetB*, *tetW*, *sulI*, and *sulII* are located on plasmids (Guo et al., 2017; Roberts & Schwarz, 2017; Rodríguez-Martínez et al., 2011), *tetA*, *tetB*, and *tetQ* are associated with conjugative transposons, and *sulI* is additionally linked with the complex class 1 integron gene (*intl1*) (Roberts, 2002, 2012). MGEs and their association with prominent bacterial phyla could facilitate the colonization of ARGs by commensal and pathogenic bacteria in wildlife (Fernandes et al., 2022; Rybak et al., 2022). A study of *L. longicaudis* gut bacteria in fecal samples from the Peñas Blancas River identified these four prominent bacteria phyla, including Campylobacterota and Deinococcota (Vargas Calvo, 2020). In the same way, other authors detected antibiotic resistance to beta-lactams (amoxicillin/clavulanate, ampicillin, penicillin), phenicols (chloramphenicol), macrolides (clindamycin), aminoglycosides (gentamicin), quinolones (enrofloxacin), tetracyclines, and vancomycin in *Enterococcus* spp. (Firmicutes), a common commensal gut bacteria in river otters (Semedo-Lemsaddek et al., 2018).

Although *L. longicaudis* from the Peñas Blancas River prefers to inhabit areas with high foliage (bushes, logs, and trees) and a wider river basin (Navarro-Picado et al., 2017), the detection of ARGs within their feces demonstrates the influence of anthropic activities that surround the river basin, and their potential to harbor, transfer, and potentially disrupt their

normal gut microbiome with resistance genes (Dolejska & Literak, 2019). This situation has been observed in other Costa Rican wildlife species, such as jaguars (*Panthera onca*), pumas (*Puma concolor*) (Angulo et al., 2023), and two-finger and three-finger sloths (*Choloepus hoffmanni* and *Bradypus variegatus*) (Fernandes et al., 2022). 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.

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 to protected areas (Bosque Eterno de los Niños) where no anthropic activities occur. 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 towards the natural environment. Future projects can benefit from our finding 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 (McEwen & Collignon, 2018; Ministerio de Salud, 2019).

L. longicaudis is a top predator in their ecosystem, and detecting ARGs in their feces might also indicate the presence of resistance 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 (Semedo-Lemsaddek et al., 2018). It´s also important to establish that the interactions human-animal-environment is too dynamic to limit the ARGs accumulation and dispersal to wildlife. Therefore, we propose future studies to monitor all possible sources of ARG contamination in the river and the presence of resistance genes within water and river otters' preferred prey.

6. DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7. DATA AVAILABILITY

Data will be made available on request.

8. ACKNOWLEDGMENTS

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XVI. ARTÍCULO 2: Pesticide contamination and antimicrobial resistance: Two threats to the Neotropical Otter (*Lontra longicaudis*) in the Peñas Blancas River Watershed

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Manuscrito preparado con el formato de la revista:

Enviromental Research

Pesticide contamination and antimicrobial resistance: Two threats to the Neotropical Otter (*Lontra longicaudis*) in the Peñas Blancas River Watershed

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ABSTRACT:

Since the introduction of synthetic pesticides, their use in agricultural practices has significantly increased productivity. Unfortunately, years of exposure to these chemicals have triggered resistance to pests and pathogens. The effects of pesticides on enhancing antimicrobial resistance (AMR) are poorly understood, but they can promote the expression and acquisition of antimicrobial-resistance genes (ARGs), especially within dense bacterial communities in residual waters from anthropogenic activities, with unknown consequences for wildlife. River otter populations in the Peñas Blancas watershed, Costa Rica, are exposed to residues from various human activities, including agriculture. This study aimed to detect the presence of pesticides in water samples in joint with ARGs in otter feces across the river basin during 2022. Six water samples (four from the middle and two from the upper basin) were analyzed for pesticides and eight otter fecal samples (six from the middle and two from the upper basin) for ARGs. We detected 13 types of pesticides (herbicides, insecticides, fungicides, and multi-target pesticides) and seven different ARGs *(qrnS, tetA, tetB, tetQ, tetW, sulI,* and *sulII*). Detected pesticides included chlorpyrifos and ethoprophos, which are regulated in Costa Rica. Chlorpyrifos, diazinon, diuron, imidacloprid, and terbutryn have been reported to promote antimicrobial resistance when bacteria are exposed at the same time to sub-lethal levels of antibiotics. Besides, it is also worrisome the high levels of ethoprophos (0.67 ng/L) found in the upper basin and that one fecal sample showed four different ARGs (*tetA, tetB, sulI,* and *sulII*). Our results highlight the agricultural practices in the Peñas Blancas watershed and the potential environmental risks to wildlife, not only because of the dangers that pesticides pose to non-targeted individuals but also by their potential impact on human and animal health due to their role in the increasing AMR problem.

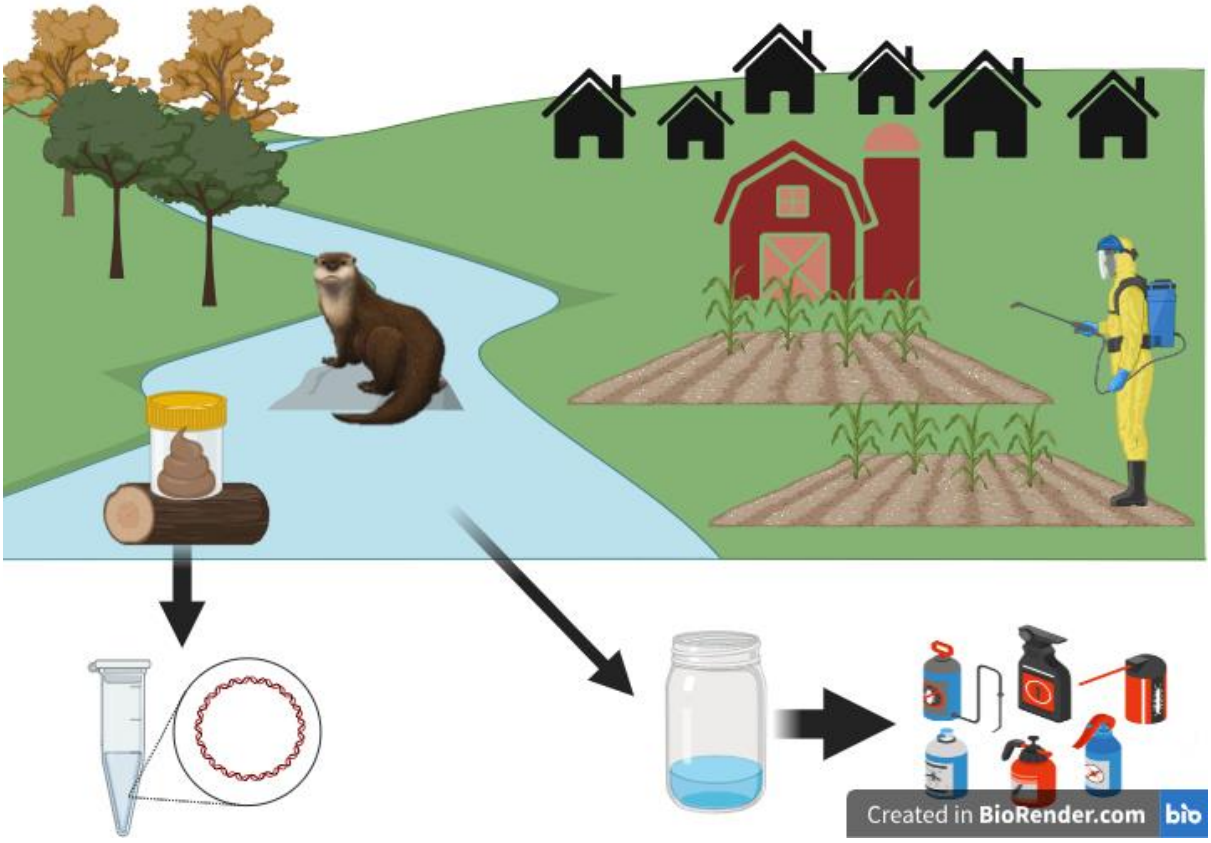
KEYWORDS:

Agriculture, anthropogenic activities, antimicrobial-resistance genes, aquatic environment, exposure, and pesticides.

HIGHLIGHTS:

- High levels of ethoprophos in waters and ARGs in otter feces near a protected area.
- Pesticides with restricted use in Costa Rica found near agricultural lands.
- Otter feces found in a pesticide-contaminated river with high diversity of ARGs.

GRAPHICAL ABSTRACT



INTRODUCTION:

Since the development of synthetic chemical pesticides in the 1940s, the use of pesticides to control pests—including insects, rodents, fungi, weeds, and bacteria—has significantly boosted agricultural productivity (Bernardes et al., 2015; Lykogianni et al., 2021). However, their extensive use has also been implied in the growing resistance among pests and pathogens (Amezian et al., 2024; Kole et al., 2019). While international organizations such as the Insecticide Resistance Action Committee (*IRAC,* 2024), Herbicide Resistance Action Committee (*HRAC,* 2024), and Fungicide Resistance Action Committee (*FRAC,* 2024) provide valuable and public information on pesticide resistance, there is a significant gap in addressing another critical issue: the impact of pesticides on the development of antimicrobial resistance (AMR).

AMR consists of the adaptation process in which microorganisms (bacteria, fungi, parasites, and viruses) develop mechanisms against antibiomicrobials (Christaki et al., 2020; O'Neill, 2016; Tang et al., 2023). This problem has been considered one of the top 10 public health threats facing humanity, and its increasing development and spreading is now considered a 'human-made' problem (WHO, 2024; 2021).

The development and spread of antibiotic-resistance genes (ARGs) induced by the interaction of bacteria with non-antibiotic stressors from pesticides can contribute to the evolution of antibiotic-resistant bacteria (ARB) (Qiu et al., 2022). Pesticides can promote the selection and emergence of multi-drug resistance (MDR), resistance towards different antibiotic families, cross-resistance (tolerance to antibiotics and heavy metals), and enhance the acquisition of ARGs by horizontal gene transfer (HGT) via conjugation between bacteria (Curutiu et al., 2017; Qiu et al., 2022).

Although there is information stating that agriculture contributes less to the development of AMR compared to other anthropogenic activities, such as those in the medical field, it still plays a role in the emergence of resistant bacteria due to the action of biocides (Curutiu et al., 2017; Miller et al., 2022). Furthermore, agricultural activities often occur near other significant pathways of AMR dissemination, such as land spreading of animal manure, sewage sludge, and aquaculture. These practices can create environments where dense bacterial communities can thrive and exchange ARGs, thereby increasing the risk of their selection and dissemination (Christaki et al., 2020; Singer et al., 2016).

Residues from anthropogenic areas containing pesticides, ARB, and ARGs can end up in aquatic environments like rivers and lakes through diverse hydrological and mobilization processes, such as rain, evaporation, sedimentation, filtration, volatilization, water drift, and runoff (Amarasiri et al., 2020; Curutiu et al., 2017; Gwenzi et al., 2020). Additionally, pesticide degradation into persistent metabolites has been associated with environmental deterioration (Tudi et al., 2022), like decreased water and solid quality and negative interaction with non-targeted organisms (e.g., embryonic malformation and alteration of the animal microbiome) (Edwards, 1993; Garcês et al., 2020; Syromyatnikov et al., 2020). And, ARG-contaminated water streams could lead to decreased buffer zones, increasing the resistance in the microorganisms and promoting wildlife to become ARG vectors (Kraemer et al., 2019; McEwen & Collignon, 2018).

The Peñas Blancas river watershed, located in Costa Rica, is characterized by its diverse agricultural (e.g., coffee, ornamental plants, fruits, and tubers) and livestock (e.g., cattle, pig, poultry, and aquaculture) activities (Inder, 2015, 2016), which could serve as exposure routes

of pesticides, antibiotics, ARGs, ARBs, and several other contaminants. This exposure could lead to a still unknown health hazard in protected areas, and its wildlife.

Monitoring the presence, dispersal, and diversity of pesticides and ARGs represents the first step toward developing and implementing programs focused on reducing environmental biocides and AMR, which is essential for wildlife conservation strategies. Our objective consisted of identifying and quantifying pesticides in the water of the Peñas Blancas River watershed in Costa Rica and detecting the presence of ARG in the feces of the aquatic top predator, the neotropical otter (*Lontra longicaudis*).

Neotropical otters have been used as sentinels for aquatic systems health and ARG dissemination, as they could be exposed to contaminants within the water as a result of the anthropogenic activities near the river (Dias et al., 2019). Previously it was identified that otters can have different ARGs in their feces (Guizado-Batista, 2024; manuscript in process of publication). However, at the time of that study, it was unclear if the presence of pesticides could explain those results, as was theorized in other studies (Angulo et al., 2023; Blanco-Peña et al., 2024; Vargas-Villalobos et al., 2024).

METHODOLOGY:

Otter feces and water sampling

During 2022, fecal samples from otters and water samples were collected during three seasons, dry (December to March), rainy (May to October), and transition (April and November). We divided the Peñas Blancas river into three areas according to the anthropogenic activities that take place across the watershed: the upper river basin, with

almost no human activities and near a protected area (Children's Eternal Rainforest), while the middle and lower river basins constituted with diverse anthropogenic areas.

Otter feces were located by walking along the river, searching for their latrine, which are rocks and logs used to demarcate their territory and for social interaction (Oldham & Black, 2009). Feces samples were collected in 50 mL falcon tubes and water samples utilizing glass bottles of 1 L, at the same water stream.

All samples were labeled with their respective codes, dates, and GPS coordinates, and kept at 4°C in the field and at -20°C in the camp base until transported to the Instituto Regional de Estudios en Sustancias Tóxicas, Universidad Nacional (IRET-UNA). Sampling and laboratory procedures were performed 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).

Pesticides analysis in water:

Water samples were extracted using the solid-phase extraction (SPE) method, employing 200 mg Isolute ENV+ cartridges (Biotage). Before their extraction, the SPE cartridges were sequentially conditioned with 5 mL of ethyl acetate, 10 mL of methanol, 10 mL of Milli-Q water, and 5 mL of methanol. Subsequently, 1000 mL of the sample was passed through the cartridge, followed by a drying process. Elution was performed using 5 mL of ethyl acetate followed by 5 mL of methanol, and the eluates were concentrated to a final volume of 0.5 mL of methanol: water (4:6).

Pesticide quantification was carried out using an ACQUITY H-CLASS UPLC chromatographic system (Waters Corp, Milford, MA, USA) coupled with a triple quadrupole Xevo TQ-S-micro-MS mass spectrometer (Waters Corp., Milford, MA, USA), equipped with a UniSpray ionization interface (US), and operated in positive mode. Instrumental parameters were optimized for each compound. A UPLC BEH C18 column was used with a temperature set at 50°C. The mobile phase consisted of solvent A (H_2O-MeOH 95/5 with 0.1% formic acid) and solvent B (NH_4-MeOH with 0.1% formic acid) at a 0.4 mL/min flow rate. An injection volume of 2 μL was used. Quantification was performed using a calibration curve comprising 99 pesticides at ten concentration levels ranging from 0.2 ng/mL to 350 ng/mL. The analysis was conducted at the Pesticide Residue Analysis Laboratory (LAREP), Regional Institute of Studies on Toxic Substances, in Costa Rica. Substances detected below the reporting limit (RL) had their concentrations reported at 10% of the RL.

Antibiotic resistance genes analysis in fecal samples:

DNA extraction was performed with the QIAamp DNA Stool Mini Kit (QIAGEN) following the manufacturer's instructions. To confirm that the fecal samples originated from *L. longicaudis*, a taxonomic analysis of the sequences of the mitochondrial control region was conducted using polymerase chain reaction (PCR). This process employed primers ProL (5′-CACCACCAACACCCAAAGCT-3′) and DLH (5′-CCTGAAGTAAGAACCAGATG-3′) (Cassens et al., 2000; Pérez-Haro et al., 2005; Valqui, 2010).

Bacteria presence and ARGs detection were done using the quantitative polymerase chain reaction (qPCR) through the CFX96 Touch Real-Time PCR Detection System, CFX96, Bio-

Rad Laboratories, Hercules, CA, USA), and SYBR Green protocols (Jiang et al., 2013). Bacteria-positive samples were identified as those with a threshold cycle (Ct) of less than 25 for the 16S rRNA gene (Villalpando et al., 2018). ARGs detection involved analyzing the presence of 12 ARGs grouped into seven antibiotic families (Table 1). Samples were considered ARG-positive if they had a Ct of less than 30.

Table 1: Antibiotic resistance genes selected for river otter feces analysis within the Peñas Blancas River watershed. Genes are grouped according to their antibiotic family, with their respective primer sequences and references.

Gene	Antibiotic Family	Primer ID	Sequence (5'-3')	Reference
***bla*$_{CTX-M}$**	Beta-Lactams	RTCTXM-F	CTATGGCACCACCAACGATA	(Marti et al., 2013)
		RTCTXM-R	ACGGCTTTCTGCCTTAGGTT	
dfrA12	Diaminopyrimidines	dfr12s-f	GAGCTGAGATATACACTCTGGCACT	(Grape et al., 2007)
		dfr12s-r	GAGCTGAGATATACACTCTGGCACT	
catII	Phenicols	C-2	GATTGACCTGAATACCTGGAA	(Yoo et al., 2020)
		C-R	CCATCACATACTGCATGATG	
erm(B)	Macrolides	erm(B)-1	CCGAACACTAGGGTTGCTC	(Di Cesare et al., 2013)
		erm(B)-2	ATCTGGAACATCTGTGGTATG	
qnrS	Quinolones	qnrSrtF11	GCAAGTTCATTGAACAGGGT	(Marti et al., 2013)
		qnrSrtR11	TCTAAACCGTCGAGTTCGGCG	
sulI	Sulfonamides	sul1-FW	CGCACCGGAAACATCGCTGCAC	(Luo et al., 2010)

Gene	Antibiotic Family	Primer ID	Sequence (5'-3')	Reference
		sul1-RV	TGAAGTTCCGCCGCAAGGCTCG	
sulII		sul2-FW	TCCGGTGGAGGCCGGTATCTGG	(Luo et al., 2010)
		sul2-RV	CGGGAATGCCATCTGCCTTGAG	
tetA	Tetracyclines	tetAS-F	TCAATTTCCTGACGGGCTG	(Keenum et al., 2022)
		tetAS-R	GAAGCGAGCGGGTTGAGAG	
tetB		tetBGK-L2	CGCCCAGTGCTGTTGTTGTC	(Goswami et al., 2008)
		tetBGK-R2	CGCGTTGAGAAGCTGAGGTG	
tetQ		TetQ-FW	AGAATCTGCTGTTTGCCAGTG	(Aminov et al., 2001)
		TetQ-RV	CGGAGTGTCAATGATATTGCA	
tetW		TetW-FW	GAGAGCCTGCTATATGCCAGC	(Aminov et al., 2001)
		TetW-RV	GGGCGTATCCACAATGTTAAC	
tetY		TetY-FW	ATTTGTACCGGCAGAGCAAAC	(Aminov et al., 2001)
		TetY-RV	GGCGCTGCCGCCATTATGC	

The ARGs quantification was calculated through the following formula:

log10(ARG%) =2 + 0,33(Ct16s - CtgenARG)*

This formula helped establish the hypothetical percentage of bacteria each gene presented for the ARGs percentage load. 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 (Angulo et al., 2023; Blanco-Peña et al., 2024; Nieto-Claudin et al., 2019; Sacristán et al., 2020; Vargas-Villalobos et al., 2024).

RESULTS:

A total of 19 otter feces and 6 water samples were analyzed. Three fecal samples came from the upper basins: 1 in Children's Eternal Rainforest (BeN), and 2 at Hydroelectric dam (Pr ICE). At the middle basins, 11 feces were obtained in Burro Brujo river (PBb), 3 from Chachaguita river (Chta), and 2 in La Lucha (Lu Pk).

Water sample's location were as such: 2 from the upper basins (1 from BeN, and 1 in Pr ICE), and 4 at the middle watershed (1 at Lu Pk, and 3 from PBp). No feces were found at the lower basins; therefore, we didn't use the pesticide results from the water samples collected in this area. Fecal and water samples distribution across the Peñas Blancas river can be visualized in Figure 1.

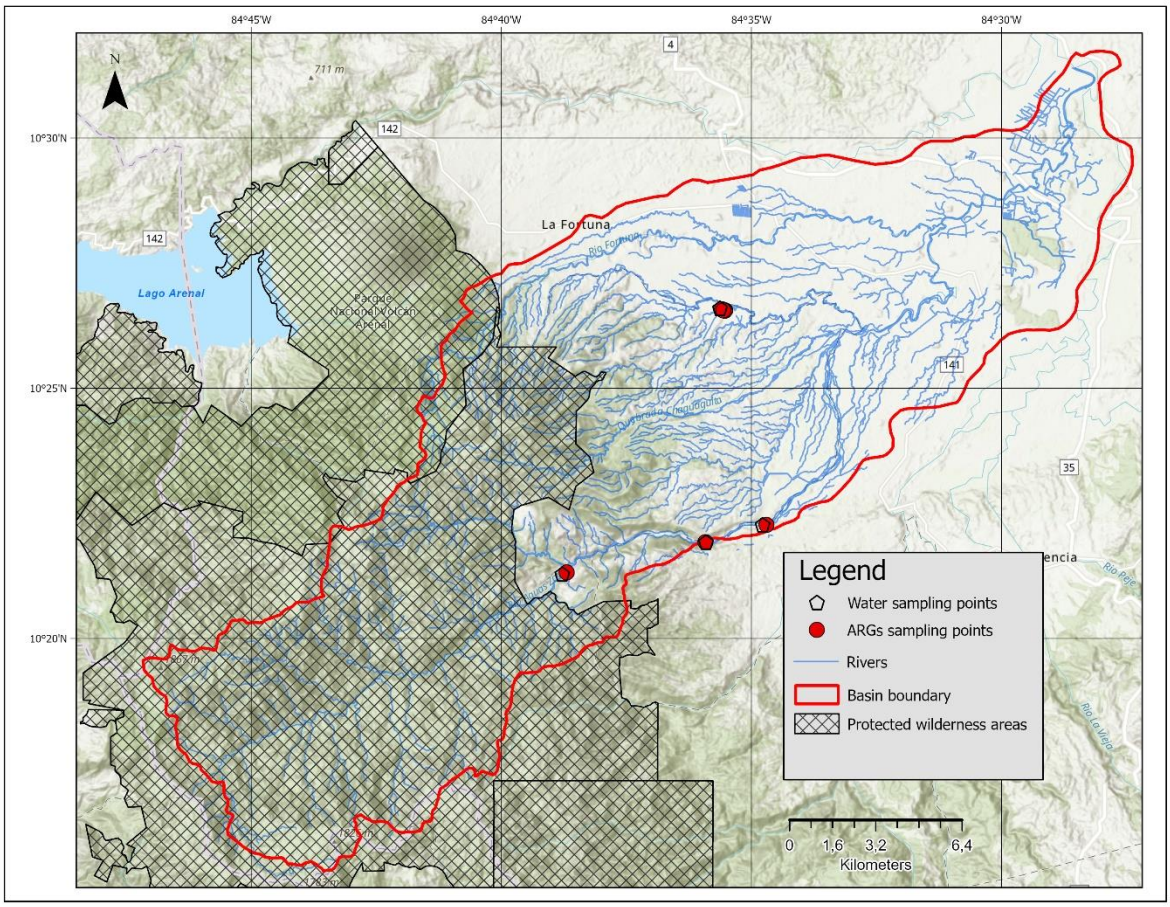


Figure 1: Water samples and ARG-positive otter feces location across the Peñas Blancas river basin, 2022.

Pesticide detection in water:

In total, 13 different types of pesticides, as well as caffeine, were found in the water samples (Table 2 and Figure 2). All six samples had at least three types of pesticides. The two samples from the upper basin had the least amount of pesticides diversity: BeN showed ametryn (0.11 ng/L), diuron (0.11 ng/L), and ethoprophos (0.67 ng/L), while Pr ICE contained diazinon (6.23 ng/L), metalaxyl (0.11 ng/L), and terbutryn (0.32 ng/L). These 6 pesticides were also detected in waters from the middle basins. No caffein was detected in samples from the upper

basins. BeN concentrations of ametryn and diuron were low when compared with does detected in the 3 samples from PBb and the one from Lu Pk, while ethoprophos had similar values with Lu Pk. Diazinon levels in Pr ICE sample had concentration levels similar to the highest values detected in Lu Pk sample, and both metalaxyl and terbutryn levels were close to those in PBb and Lu Pk.

In total, 13 different pesticides were detected at the middle river basins, with one sample from PBb containing 11: ametryn, cadusafos, carbendazim, clothianidin, diazinon, diuron, imidacloprid, metalaxyl, oxamyl, pyraclostrobin, and terbutryn. It also displayed the highest concentration of diuron (31.62 ng/L), ametryn (4 ng/L), imadacloprod (1.37 ng/L), and metalaxyl (0.66 ng/L). The Pr PK sample got 6 pesticides, and lowest concentration levels from all the middle basins samples, except for diazinon, with 7 ng/L.

Table 2: Concentration levels of pesticides (ng/L) detected in water samples collected in Peñas Blancas River Watershed, Costa Rica, during 2022. Sites abbreviations: Children's Eternal Rainforest = BeN; Hydroelectric dam ICE = Pr ICE; La Lucha = Lu Pk; and Burro Brujo river = PBb.

Site abbreviation	BeN	Pr ICE	PBb	PBb	PBb	Lu-Pk
Basin subdivision	Upper	Upper	Middle	Middle	Middle	Middle
Collection date (d/m/y)	30/05/2022	22/11/2022	30/05/2022	12/07/2022	22/11/2022	30/05/2022
Pesticides						
Ametryn	0.11	n.d.	4.00	0.11	0.16	0.11
Caffeine	n.d.	n.d.	12.96	0.55	26.76	n.d.
Caudosafos	n.d.	n.d.	0.11	n.d.	n.d.	0.11
Carbendazim	n.d.	n.d.	3.09	n.d.	3.56	n.d.
Clothianidin	n.d.	n.d.	0.44	n.d.	n.d.	n.d.
Chlorpyrifos	n.d.	n.d.	n.d.	0.81	n.d.	n.d.
Diazinon	n.d.	6.63	1.00	n.d.	1.41	7.00
Diuron	0.11	n.d.	31.62	14.32	7.75	0.73
Ethoprophos	0.67	n.d.	n.d.	0.05	n.d.	0.73
Imidacloprid	n.d.	n.d.	1.37	n.d.	0.78	n.d.
Metalaxyl	n.d.	0.11	0.66	0.30	0.11	0.45
Oxamyl	n.d.	n.d.	0.22	n.d.	n.d.	n.d.
Pyraclostrobin	n.d.	n.d.	0.27	n.d.	n.d.	n.d.
Terbutryn	n.d.	0.32	0.11	n.d.	n.d.	n.d.

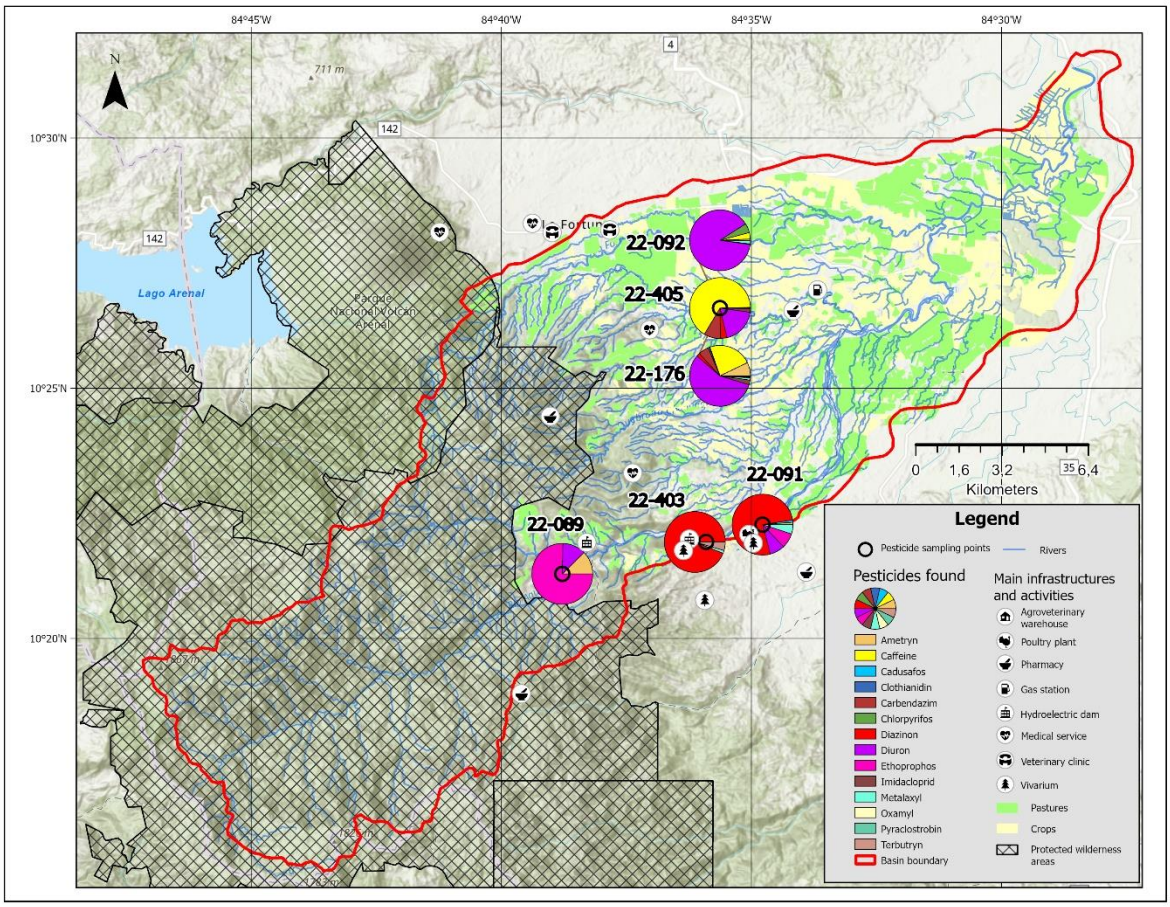


Figure 2: Map displaying all six water sampling points, their proportions in relation to all pesticides detected, and anthropogenic activities nearby. Note: samples 22-092 and 22-176 were collected in the same site.

Antibiotic Resistance Genes detection in otter feces:

Eight fecal samples were ARG-positive for at least one gene (42%). Genes detected belonged to the following antibiotic families: quinolones (*qrnS*), tetracyclines (*tetA*, *tetB*, *tetQ*, and *tetW*), and sulfonamides (*sulI* and *sulII*). Their distribution was as follows: two samples (25%) from the upper basin (one in the BeN and the other one, in Pr ICE) and six samples (75%) from the middle basin (one in Lu Pk and five in PBb) (Table 3). No beta-lactam

(*bla*$_{CTX}$), diaminopyrimidines (*dfrA12*), phenicol (*catII*), and macrolide (*erm(B)*) genes were detected.

Table 3: Antibiotic resistance genes detected from *Lontra longicaudis* feces across the Peñas Blancas river watershed collected in 2022. Minimum (Min) and maximum (Max) values, number (#), and percentage (%) of ARG-positive samples obtained from the upper and middle basin subdivision (n.d. = not detected).

ARGs	Upper basin (n=2)				Middle basin (n=6)			
	Min	**Max**	**Positives**		**Min**	**Max**	**Positives**	
			#	**%**			**#**	**%**
qnrS	n.d.	n.d.	-	-	-1.32	-0.96	2	33
tetA	-0.49		1	50	-0.77		1	17
tetB	-0.91	-0.82	2	100	-1.31	-0.61	2	33
tetQ	n.d.	n.d.	-	-	-1.57	0.83	3	50
tetW	n.d.	n.d.	-	-	-0.14		1	17
sulI	-0.82		1	50	-1.24	0.44	3	50
sulII	-1.56		1	50	-1.29	-0.25	3	50

Across the watershed, the most detected ARGs were *tetB* (20%), *sulI* (20%), and *sulII* (20%), followed by *tetQ* (15%), *qrnS* (10%), *tetA* (10%) and *tetW* (5%). Sample 2-PBBB1 (from PBb) got the highest diversity of ARGs, with five different genes: *tetB*, *tetQ*, *tetW*, *sulI*, and *sulII*. It was followed by samples 16-PBPP2 (BeN), which showed four genes: *tetA*, *tetB*, *sulI*, and *sulII*.

DISCUSSION:

Throughout 2022 we detected 13 types of pesticides across the waters from Peñas Blancas river. These pesticides act as herbicides (ametryn, diuron, terbutryn), fungicides (carbendazim, metalaxyl, pyraclostrobin), and insecticides (clothianidin, chlorpyrifos, imidacloprid). Notably, cadusafos, diazinon, ethoprophos, and oxamyl, act on multiple types of pests, including insects, nematodes, and acarids.

Ametryn, diuron, and diazinon were among the most frequently detected pesticides in Costa Rican freshwater between 2009 and 2019 (Echeverría-Sáenz et al., 2021), with our diuron concentrations exceeding 10 ng/L in two samples. Organophosphates like chlorpyrifos and ethoprophos, and carbamates such as carbendazim and oxamyl, are widely used in agriculture and are known for their neurotoxic effects and environmental persistence (Gupta et al., 2017). Chlorpyrifos and ethoprophos are highly toxic chemicals , and a severe threat to the environment and wildlife (specifically aquatic species) (Gupta et al., 2011, 2017). Additionally, their use is restricted in Costa Rica since 2007 (SFE, 2024). So, it is concerning that chlorpyrifos, carbendazim, and oxamyl were detected middle basins (PBb) where several of the otter´s fecal samples were obtained.

Ethoprophos, was detected at BeN, which is close to a protected area, in addition to PBb and Lu Pk. The presence of ethoprophos, as well as diuron and ametryn at the BeN, could suggest the misuse of these pesticides surrounding the Peñas Blancas watershed. But it is also important to establish that these chemicals are persistent in soil and water for extended periods (Carvalho, 2017; Fernández-Calviño et al., 2021; Ramasubramanian, 2013; WHO & IPCS, 1993).

Pesticide and antibiotic residues from irrigation and municipal wastewater inevitably end up in the environment and could work in conjunction to facilitate antibiotic resistance in bacteria, promoting ARB dissemination (Qiu et al., 2022). Pesticide-degrading microorganisms can use pesticides as an energy source, a nutrient source, or degrade them co-metabolically, aided by enzymes associated with antibiotic inactivation. (Ramakrishnan et al., 2019). Xing et al. (2020) showed that long-term exposure to a mixture of 23 frequently detected pesticides (including chlorpyrifos, diazinon, diuron, imidacloprid, and terbutryn) led to the emergence of significant streptomycin resistance in *Escherichia coli*. When exposed to both pesticides and sub-inhibitory levels of ampicillin, *E. coli* exhibited increased resistance to ampicillin and cross-resistance to other antibiotics, such as ciprofloxacin, chloramphenicol, and tetracycline. This selective pressure from pesticide concentrations, antibiotic types, and bacterial structural changes can enhance cell permeability, facilitate HGT, and promote overexpression of antibiotic efflux pumps, contributing to multidrug resistance in bacteria (Qiu et al., 2022; Ramakrishnan et al., 2019).

The Peñas Blancas River exhibits significant pesticide and caffeine contamination. Due to caffeine detection being related to anthropogenic influence (Causanilles et al., 2017; Wilkinson et al., 2022), it was expected to observe these high concentrations in the middle

basins, where diverse anthropogenic activities take place, such as agriculture, poultry farming, urban development, and medical facilities. This anthropogenic influence correlates with the detection of ARGs in otter fecal samples. Notably, in the less-impact upper basin, which has minimal human presence and low pesticide levels, four ARGs were found in one sample (16-PBPP2). River otters (*Lontra longicaudis*), with home ranges of 3 to 8 km and some tolerance to human activity (Gallo-Reynoso, 1989; Ortega et al., 2012), may be transporting these ARGs from the contaminated middle basin to the cleaner upper basin. This phenomenon underscores the complex interaction between wildlife movement and environmental pollution. The presence of pesticides and ARGs, even near pristine areas like the Children's Eternal Rainforest, highlights the potential of river otters as bioindicators of aquatic ecosystem health, given their exposure to contaminants in their foraging and territorial activities (Dias et al., 2019; Semedo-Lemsaddek et al., 2018).

The effect of pesticides on the antimicrobial resistance (AMR) process is still an ongoing topic of study. Although our study can´t establish that the pesticides detected are directly influencing the occurrences and prevalences of ARGs, there is still a high potential for them to enhance the expression and acquisition of ARGs. Furthermore, all ARGs we found are mobile genetic elements (MGEs), a crucial factor in HGT (Partridge et al., 2018). These MGEs are present in plasmids (*qnrS*, *tetB*, *tetW*, *sulI*, and *sulII*), are correlated with conjugative transposons (*tetA*, *tetB*, and *tetQ*), and linked with the integron *intl1* (*sulI*) (Guo et al., 2017; Roberts, 2012; Roberts & Schwarz, 2017; Rodríguez-Martínez et al., 2011). This same diversity of ARGs was detected in otters' feces from Peñas Blancas River in a previous study (Guizado-Batista, 2024), highlighting anthropogenic activities' effects on the environment and the prevalence of ARGs in *L. longicaudis*.

Future studies could use our findings to establish better regulations on pesticide use in agriculture and, consequently, reduce the presence of ARGs. It is essential to develop strategies to raise public awareness about the health consequences of pesticide runoff on aquatic species and the people living in the basin. Additionally, we encourage campaigns to enhance understanding of the potential human health threats posed by AMR, its impact on wildlife conservation, and the need to mitigate ARG dispersal into the environment.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY

Data will be made available on request.

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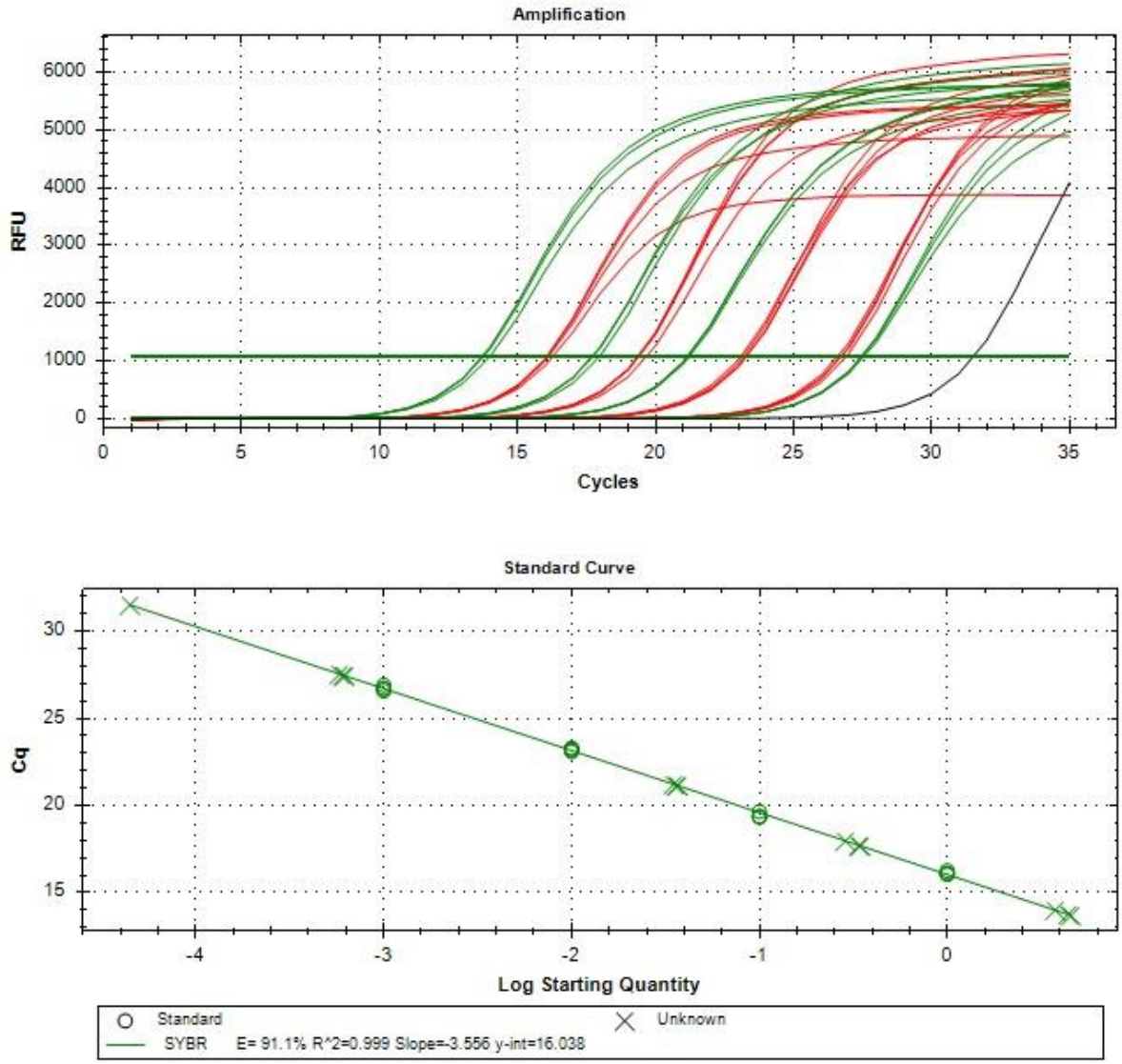
Anexo 1: Curvas de especificidad para las muestras positivas al gen 16s (1S-a) y todos los ARGs detectados (1S-b al 1S-h).

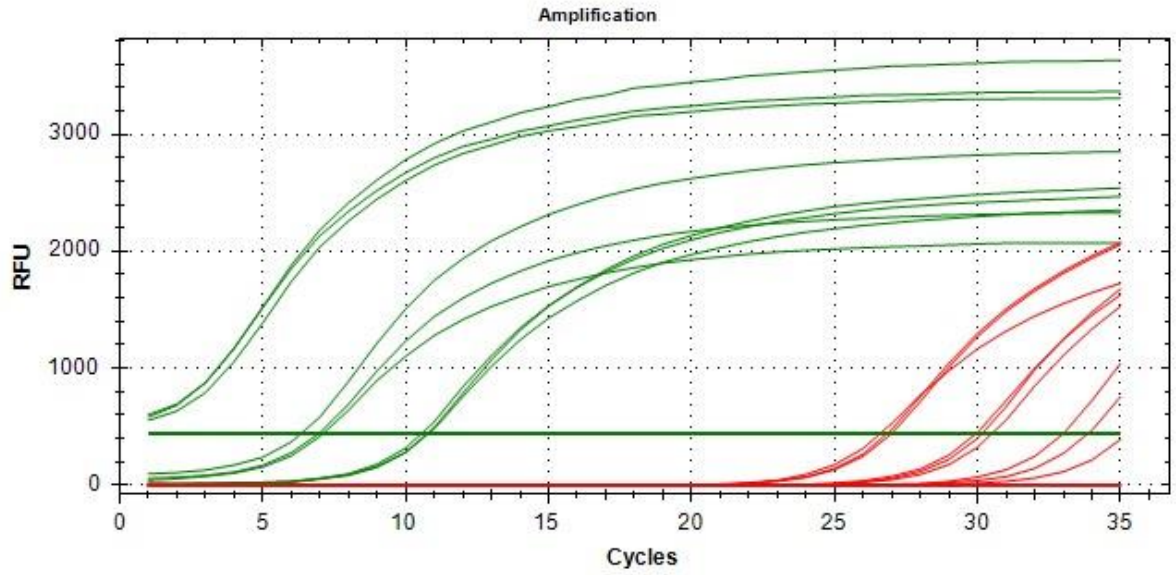
Curvas de amplificación: rojo = muestra de heces de nutria; verde = control positivo; negro = control negativo.

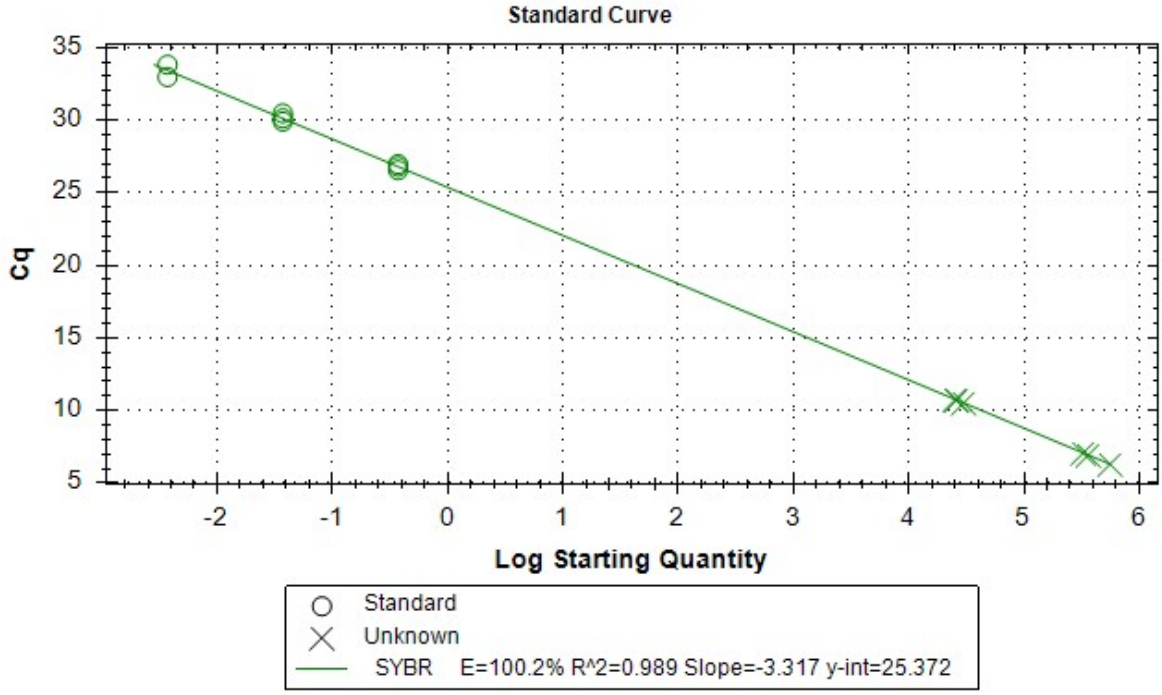
Cuadro de curvas estándar: O (estándar) = muestra de heces de nutria; X (Desconosido) = control positivo.

(A). Curva de especificidad para el gen 16s rRNA:

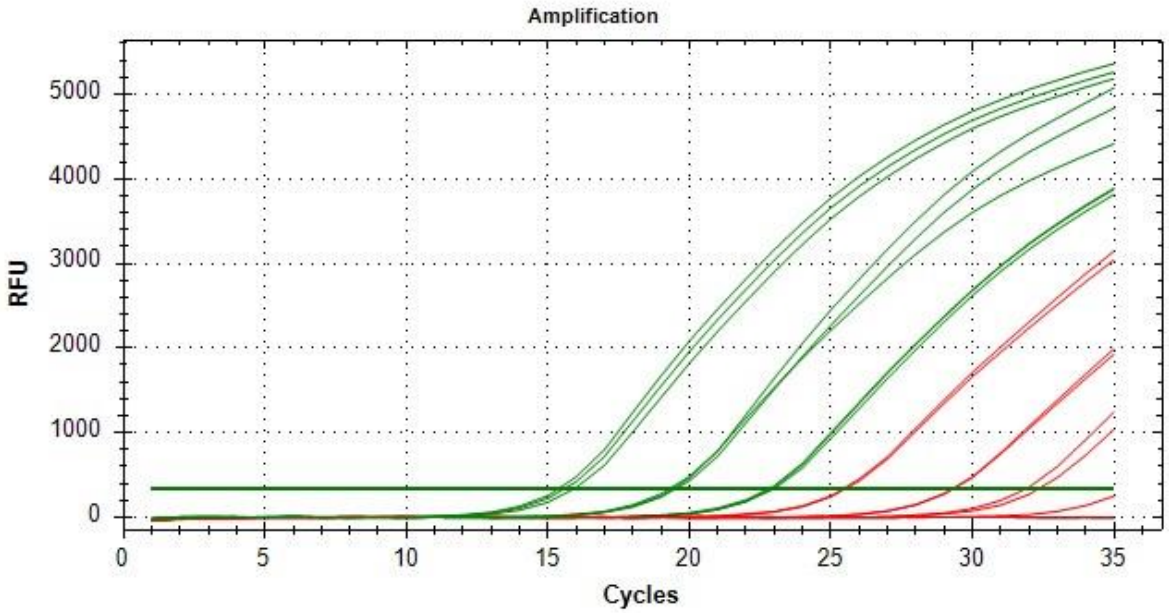


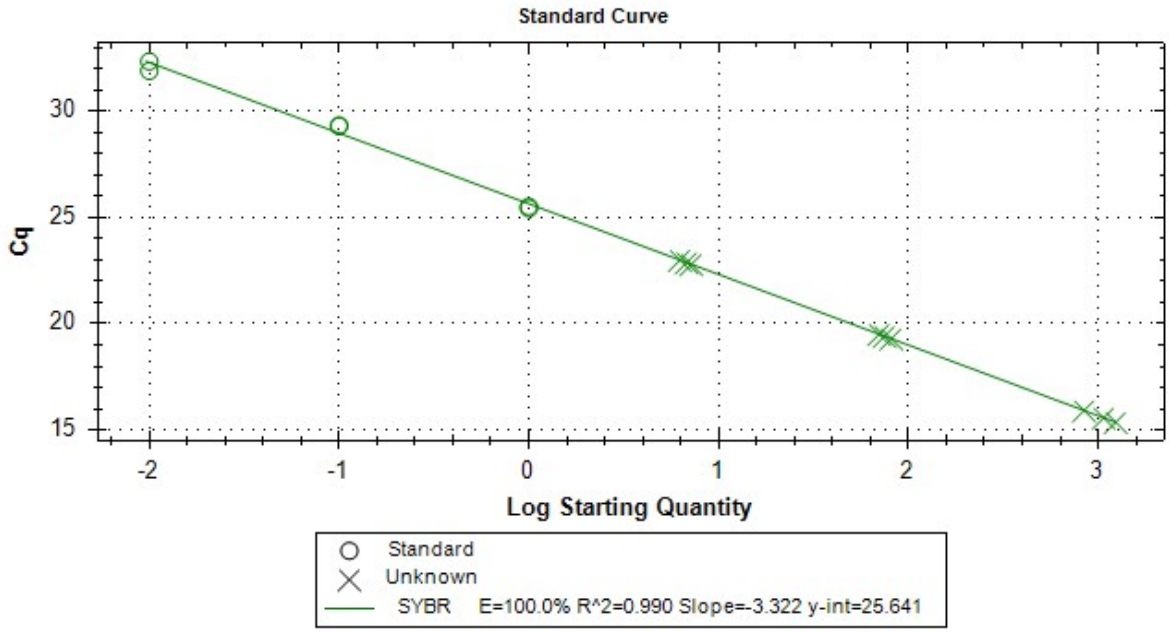
(B). Curva de especificidad para *qnrS*:



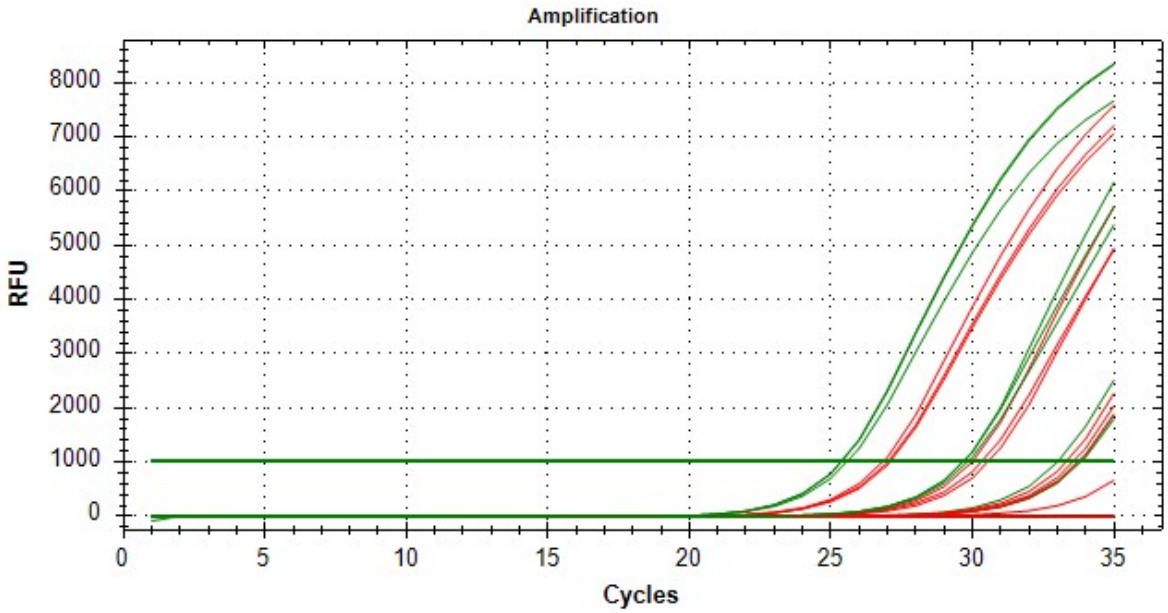


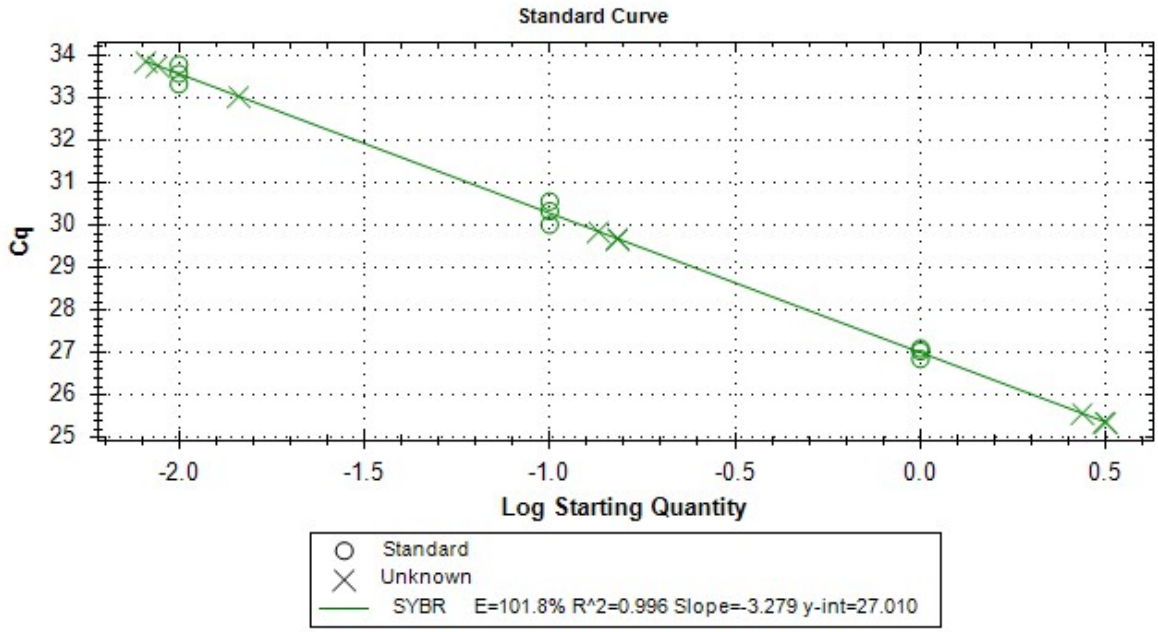
(C). Curva de especificidad para *tetA*:



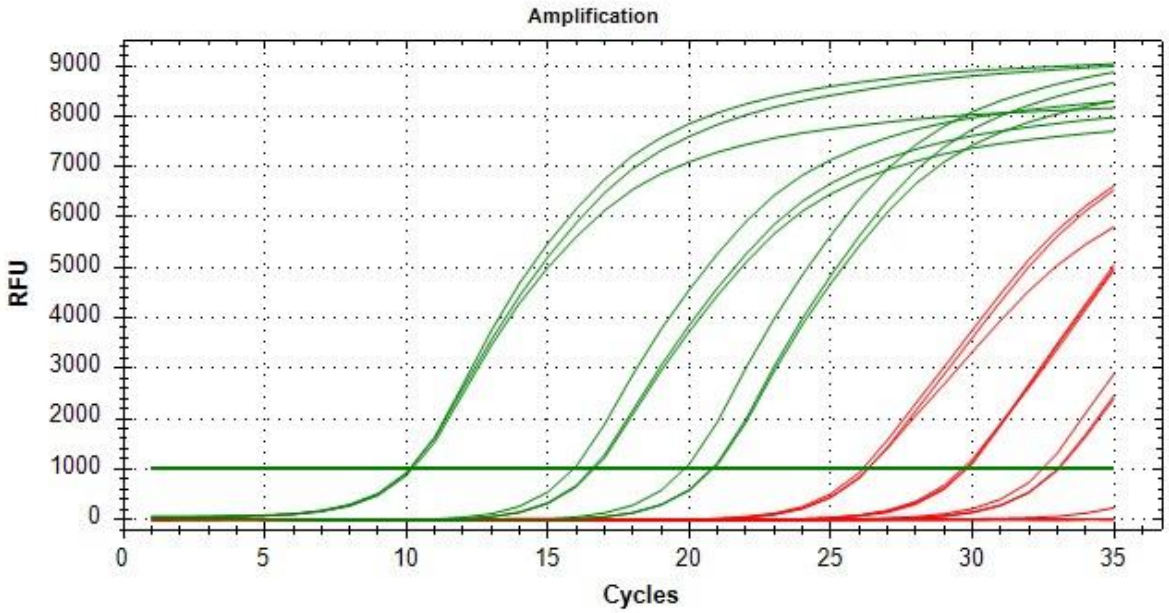


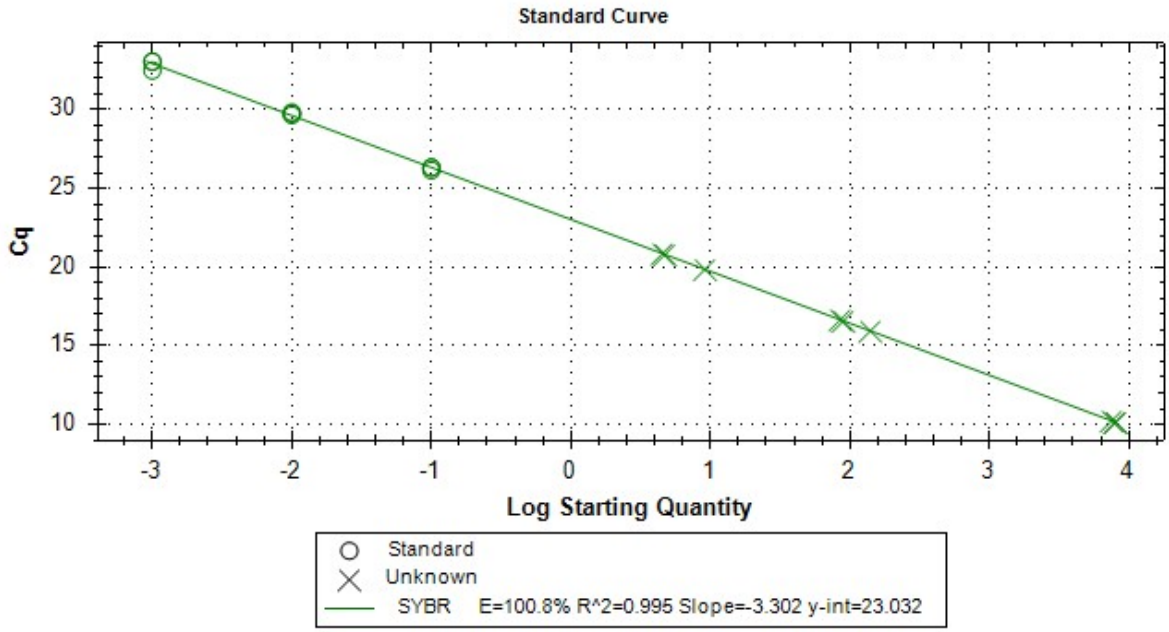
(D). Curva de especificidad para *tetB*:



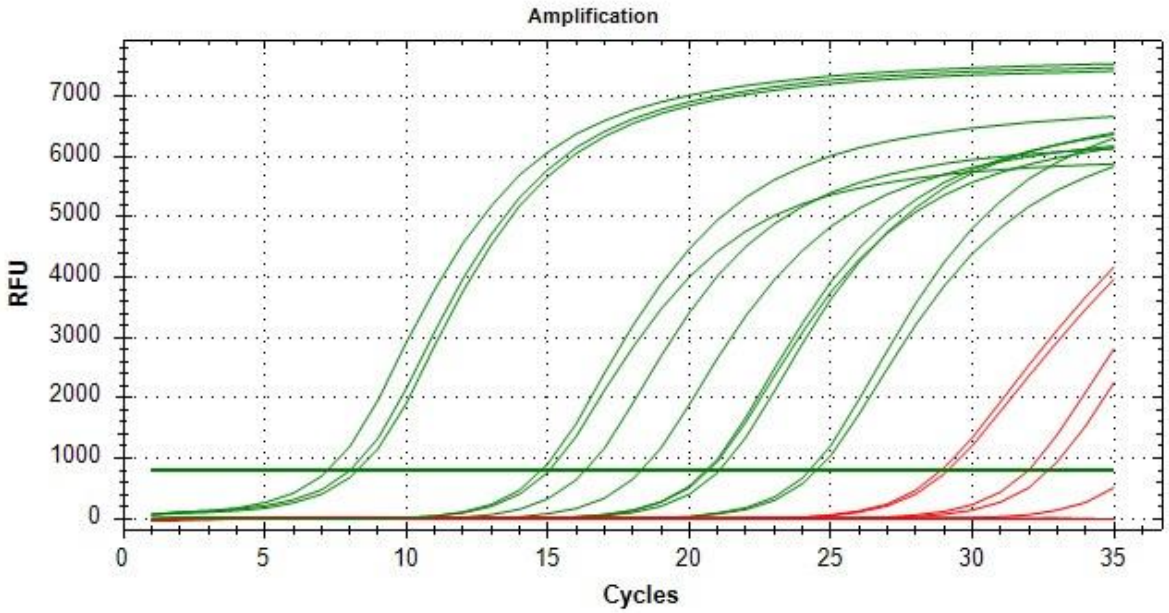


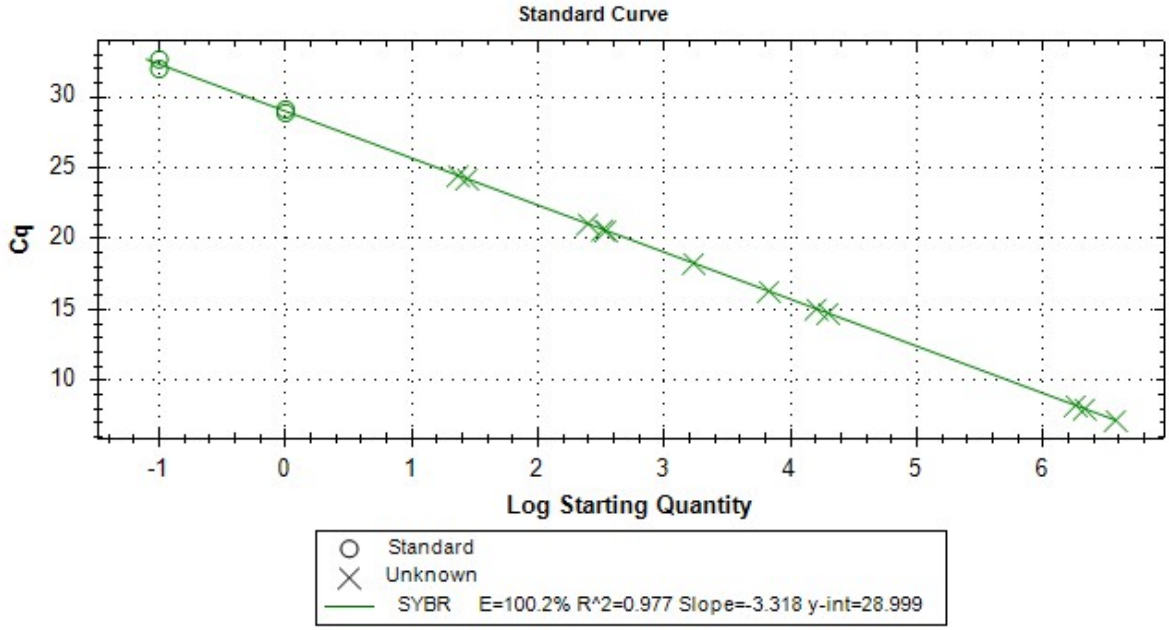
(E). Curva de especificidad para *tetQ*:



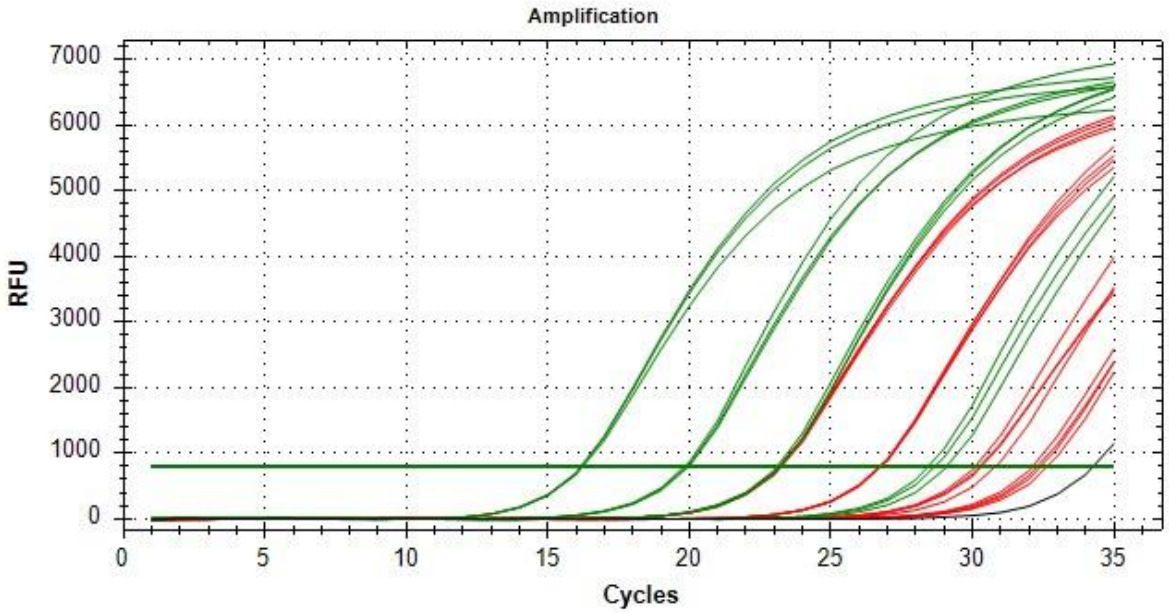


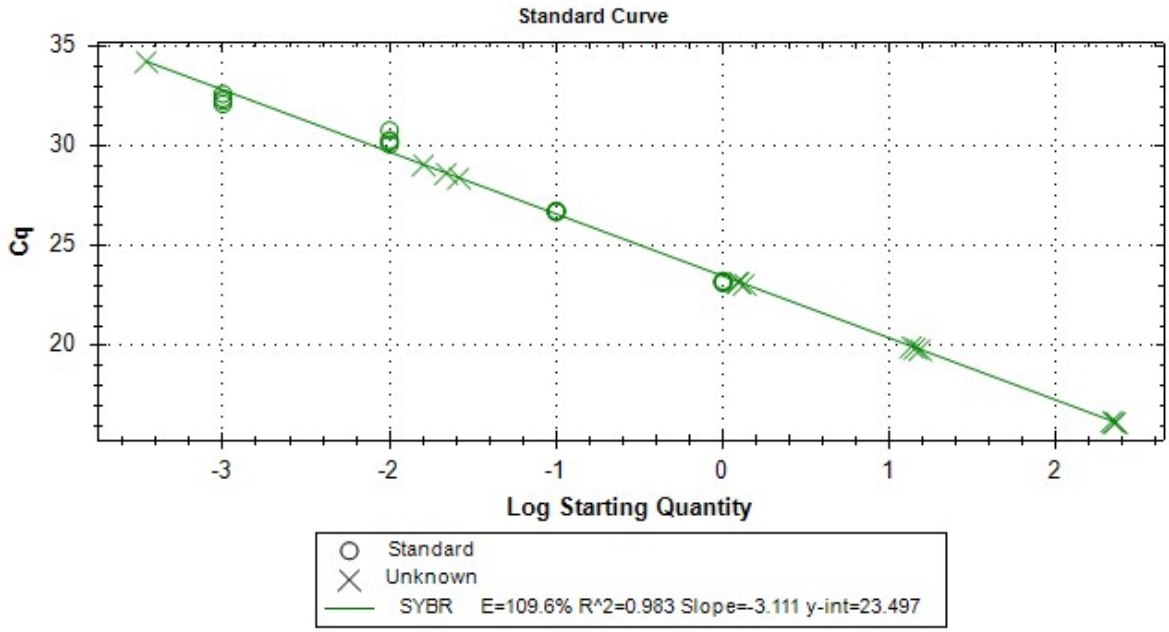
(F). Curva de especificidad para *tetW*:



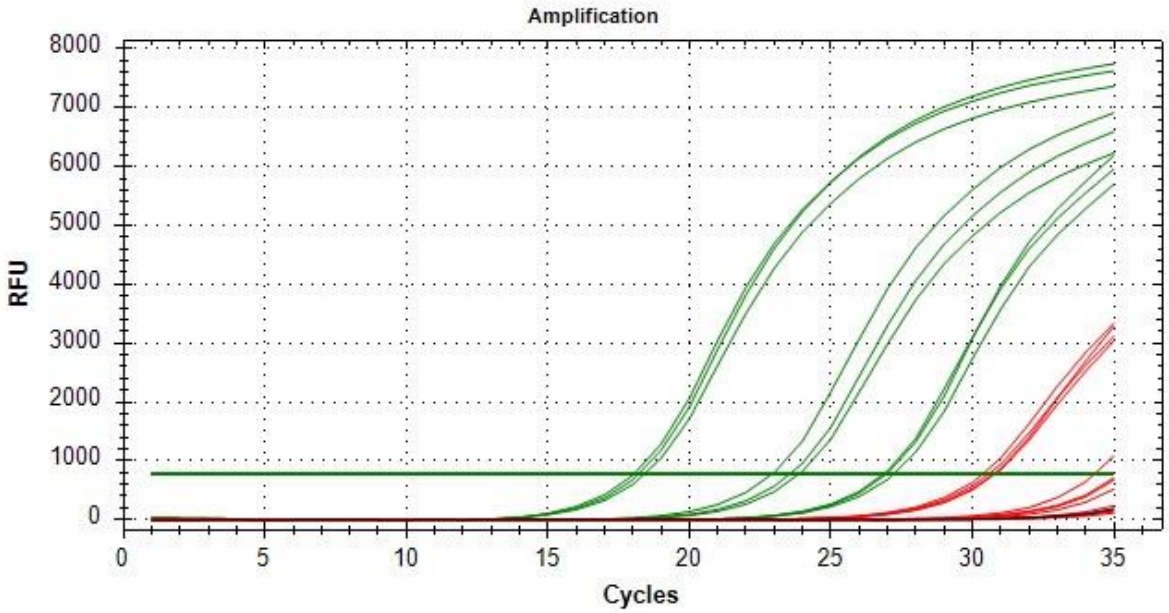


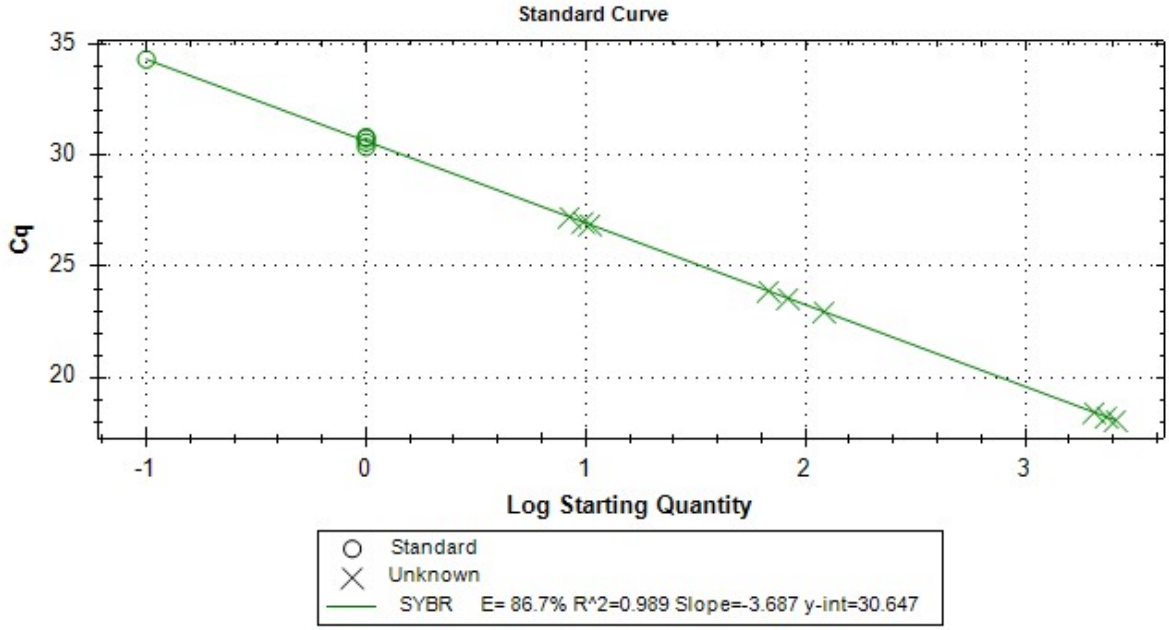
(G). Curva de especificidad para *sulI*:





(H). Curva de especificidad para *sulII*:


Amplification
RFU
8000
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
35
Cycles


Standard Curve
Cq
35
30
25
20
-1
0
1
2
3
Log Starting Quantity
Standard
Unknown
SYBR E= 86.7% R^2=0.989 Slope=-3.687 y-int=30.647

Anexo 2: secuencia de Primers utilizados para la cuantificación de los ARGs.

Gen	**Secuencia "Forward" y "Reverse" de los Primers**	**Tamaño del fragmento (pb)**	**Temperatura (°C) de Anniling**	**Referencia**
blaCTX	CTATGGCACCACCAACGATA ACGGCTTTCTGCCTTAGGTT	103	60	(Marti et al., 2013)
catII	GATTGACCTGAATACCTGGAA CCATCACATACTGCATGATG	566	63	(Yoo et al., 2003)
dfrA12	GAGCTGAGATATACACTCTGGCACT GAGCTGAGATATACACTCTGGCACT	155	60	(Grape et al., 2007)
erm(B)	CCGAACACTAGGGTTGCTC ATCTGGAACATCTGTGGTATG	139	56	(Di Cesare et al., 2013)
qnrS	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	300	62	(Marti & Balcázar, 2013)
sulI	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	158	60	(Luo et al., 2010)
sulII	TCCGGTGGAGGCCGGTATCTGG CGGGAATGCCATCTGCCTTGAG	190	58.5	(Luo et al., 2010)
tetA	TCAATTTCCTGACGGGCTG GAAGCGAGCGGGTTGAGAG	91	60	(Keenum et al., 2022)
tetB	CGCCCAGTGCTGTTGTTGTC CGCGTTGAGAAGCTGAGGTG	171	63	(Goswami et al., 2008)
tetQ	AGAATCTGCTGTTTGCCAGTG CGGAGTGTCAATGATATTGCA	169	63	(Aminov et al., 2001)

Gen	**Secuencia "Forward" y "Reverse" de los Primers**	**Tamaño del fragmento (pb)**	**Temperatura (°C) de Anniling**	**Referencia**
tetW	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	168	64	(Aminov et al., 2001)
tetY	ATTTGTACCGGCAGAGCAAAC GGCGCTGCCGCCATTATGC	181	68	(Aminov et al., 2001)

Anexo3: todas las muestras de heces de nutrias positivas para 16s rRNA (2019-2022), - fecha de recolección (d/m/a), temporada coordenadas en la cuenca del río Peñas Blancas y resultados log_{10} porcentual de cada ARG.

Las muestras con (*) indican diluciones en base a 10.

Código de Muestra	Fecha (d/m/a)	Temporada	Latitud	Longitud	Ct 16S primera extracción de ADN	Cuantificación (log10 * porcentaje de un ARG)										
						blaCTX	*ermB*	*Cat II*	*qnrS*	*tetA*	*tetB*	*tetQ*	*tetW*	*tetY*	*sul I*	*sul II*
E-PBA-3	11/09/2019	Lluviosa	10.346272	-84.655136	16.42	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PeB-1	11/09/2019	Lluviosa	10.371061	-84.589431	22.25	n.a.	n.a.	n.a.	-0.0889	n.a.	0.9605	n.a.	n.a.	n.a.	0.5843	n.a.
PeB-4	11/09/2019	Lluviosa	10.371061	-84.577275	20.32	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
For-2	25/09/2019	Lluviosa	10.466000	-84.603719	19.6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
For-5A	25/09/2019	Lluviosa	10.465967	-84.601497	21.85	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
For-5B	25/09/2019	Lluviosa	10.465967	-84.601497	21.8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
For-6	25/09/2019	Lluviosa	10.465844	-84.600936	21.89	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Paj-1	14/10/2019	Lluviosa	10.454125	-84.546122	21.65	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.0398	n.a.	n.a.	n.a.	n.a.
Paj-2	14/10/2019	Lluviosa	10.456364	-84.555814	21.82	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Bur-3A	17/10/2019	Lluviosa	10.455867	-84.630442	19.07	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Bur-3B	17/10/2019	Lluviosa	10.455867	-84.630442	15.65	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Bur-6B	17/10/2019	Lluviosa	10.456186	-84.629031	18.16	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Bur-6C	17/10/2019	Lluviosa	10.456186	-84.629031	20.37	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
For-12	22/10/2019	Lluviosa	10.466975	-84.620392	19.21	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
For-13B	22/10/2019	Lluviosa	10.467214	-84.620817	19.77	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
For-14B	24/10/2019	Lluviosa	10.470219	-84.573414	20.27	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
For-15	24/10/2019	Lluviosa	10.475247	-84.567389	20.4	n.a.	n.a.	n.a.	n.a.	n.a.	-0.7555	n.a.	n.a.	n.a.	n.a.	-1.1251
Bur-11	19/11/2019	Transición	10.446436	-84.596342	20.61	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Bur-12	19/11/2019	Transición	10.444506	-84.594203	19.4	n.a.	n.a.	n.a.	n.a.	n.a.	-1.0195	n.a.	n.a.	n.a.	n.a.	n.a.
Bur-13	19/11/2019	Transición	10.443378	-84.593336	16.3	n.a.	n.a.	n.a.	n.a.	n.a.	-0.6334	n.a.	n.a.	n.a.	-2.1184	n.a.
Bur-15	26/11/2019	Transición	10.439989	-84.578017	19.9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Bur-16	26/11/2019	Transición	10.442244	-84.551756	21.24	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PEB-7	27/1/2019	Seca	10.371267	-84.579217	18.97	n.a.	n.a.	n.a.	-1.4551	n.a.	-1.6267	n.a.	n.a.	n.a.	-0.9865	-0.7192
For-19	16/12/2019	Seca	10.473761	-84.543633	22.06	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
For-20	16/12/2019	Seca	10.473208	-84.543233	19.55	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
For-21	16/12/2019	Seca	10.472244	-84.541181	17.07	n.a.	n.a.	n.a.	n.a.	n.a.	-0.9766	n.a.	n.a.	n.a.	-2.0227	n.a.
PEB-8	17/12/2019	Seca	10.384192	-84.564564	16.96	n.a.	n.a.	n.a.	-1.9765	n.a.	n.a.	-0.3859	-1.762	n.a.	-2.0656	-2.026
PEB-10	17/12/2019	Seca	10.371275	-84.579283	19.86	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Cha-6	12/01/2020	Seca	10.409656	-84.568767	22.23	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Bur-20	18/01/2020	Seca	10.443175	-84.605186	20.21	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Paj-3	05/02/2020	Seca	10.452786	-84.574933	16.32	n.a.	n.a.	n.a.	n.a.	n.a.	-1.0492	n.a.	-2.4055	n.a.	-1.4353	-1.3759
Paj-4	05/02/2020	Seca	10.453189	-84.573922	19.05	n.a.	n.a.	n.a.	n.a.	n.a.	-1.1482	n.a.	n.a.	n.a.	-0.8908	-0.871
Paj-5C	05/02/2020	Seca	10.454417	-84.567406	18.55	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-1.5178	n.a.	n.a.	n.a.

Código de Muestra	**Fecha (d/m/a)**	**Temporada**	**Latitud**	**Longitud**	**Ct 16S primera extracción de ADN**	**Cuantificación (log10 * porcentaje de un ARG)**										
						blaCTX	***ermB***	***Cat II***	***qnrS***	***tetA***	***tetB***	***tetQ***	***tetW***	***tetY***	***sul I***	***sul II***
Bur-21	06/02/2020	Seca	10.443325	-84.593325	20.15	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Bur-23	06/02/2020	Seca	10.443144	-84.593231	16.96	n.a.	n.a.	n.a.	n.a.	n.a.	-1.7521	n.a.	n.a.	n.a.	n.a.	n.a.
Bur-24	06/02/2020	Seca	10.443078	-84.593156	15.61	n.a.	n.a.	n.a.	n.a.	n.a.	-2.2801	n.a.	n.a.	n.a.	n.a.	-2.2768
But-2	06/02/2020	Seca	10.432372	-84.598142	21.51	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
CHT-1	10/02/2020	Seca	10.430883	-84.563947	20.58	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PEB-12	10/02/2020	Seca	10.371875	-84.580089	14.37	n.a.	n.a.	n.a.	-2.9995	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-2.7487
Bur-27	23/02/2020	Seca	10.443592	-84.603661	19.84	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Bur-28	23/02/2020	Seca	10.443658	-84.600942	17.48	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-1.4485
16-PBPP2*	26/05/2022	Lluviosa	10.355331	-84.644899	18.5	n.a.	n.a.	n.a.	n.a.	-0.4948	-0.9139	n.a.	n.a.	n.a.	-0.8083	-1.5607
1-PBPP4	14/06/2022	Lluviosa	10.371204	-84.578380	17.67	n.a.	n.a.	n.a.	-1.3165	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2-PBBB1	15/06/2022	Lluviosa	10.443016	-84.593144	21.33	n.a.	n.a.	n.a.	n.a.	n.a.	-0.6103	0.8252	-0.1417	n.a.	0.3698	-0.2506
3-PBBB2	15/06/2022	Lluviosa	10.442954	-84.593100	18.83	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5-PBBB4*	15/06/2022	Lluviosa	10.442630	-84.592733	19.63	n.a.	n.a.	n.a.	n.a.	-0.772	n.a.	-0.871	n.a.	n.a.	n.a.	-1.2934
6-PBBB5	15/06/2022	Lluviosa	10.442617	-84.592729	21.32	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8-PBBB7	15/06/2022	Lluviosa	10.444144	-84.591112	23.47	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
11-PBChT1	16/06/2022	Lluviosa	10.429283	-84.565186	21.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
12-PBChT2	16/06/2022	Lluviosa	10.430960	-84.563633	19.92	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
18-PBBB10*	13/07/2022	Lluviosa	10.442565	-84.592090	19.05	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-1.5673	n.a.	n.a.	-1.2373	-0.5575
19-PBBB11	13/07/2022	Lluviosa	10.442527	-84.592174	18.32	n.a.	n.a.	n.a.	-0.9634	n.a.	-1.6069	n.a.	n.a.	n.a.	n.a.	n.a.
20-PBBB12	14/11/2022	Transición	10.443140	-84.593216	21.22	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
21-PBCht3	15/11/2022	Transición	10.433245	-84.562173	16.12	n.a.	n.a.	n.a.	n.a.	n.a.	-2.0623	n.a.	n.a.	n.a.	n.a.	n.a.
22-PBICE 1	15/11/2022	Transición	10.365390	-84.598708	20.19	n.a.	n.a.	n.a.	n.a.	n.a.	-0.8215	n.a.	n.a.	n.a.	n.a.	n.a.
24-PBBB13	16/11/2022	Transición	10.442642	-84.592742	19.67	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.4424	n.a.