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Antimicrobial resistance in Galapagos tortoises as an indicator of the growing human footprint☆

Ainoa Nieto-Claudin [a,b,c,*], Sharon L. Deem [a,c], Casilda Rodríguez [b], Santiago Cano [b], Nicolas Moity [a], Freddy Cabrera [a], Fernando Esperón [b,d]

[a] Charles Darwin Research Station, Charles Darwin Foundation, Santa Cruz, Galapagos, Ecuador
[b] Complutense University of Madrid, Veterinary Faculty, Puerta de Hierro Av, Madrid, 28040. Spain
[c] Saint Louis Zoo Institute for Conservation Medicine, One Government Drive, Saint Louis, MO, 63110. USA
[d] INIA-CISA. Algete-El Casar Road, Valdeolmos, 28130. Spain

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ABSTRACT

Antimicrobial resistance has become one of the main public health threats worldwide with anthropogenic activities driving the spread of resistance. Understanding and combatting the spread of resistant bacteria is a top priority for global health institutions, and it is included as one of the main goals of the One Health initiative. Giant tortoises (*Chelonoidis* spp.), some of the most iconic species on Earth, are widely distributed across the Galapagos archipelago and are thus perfect candidates to test the hypothesis that wildlife species in the Galapagos carry antimicrobial resistant genes (ARGs) associated with human activities. We sampled a total of 200 free-living Galapagos tortoises from western Santa Cruz Island (*C. porteri*), the most human-populated island of the archipelago, and 70 tortoises (*C. vandenburghi*) from the isolated Alcedo Volcano on Isabela Island, a natural area with minimal human presence. Fecal samples were analyzed by quantitative PCR for a panel of 21 ARGs conferring resistance for eight antimicrobial classes. We found ARGs in both Santa Cruz and Alcedo Volcano giant tortoises; however, both qualitative and quantitative results showed higher loads of ARGs in tortoises inhabiting the human modified environments of Santa Cruz. Moreover, Santa Cruz tortoises sampled in higher human-modified landscapes (i.e., farmlands and urban areas) presented a higher number of ARGs, antimicrobial classes, and multi-resistant microbiomes than those from less anthropized areas within the same island. Our findings suggest that human activities in Galapagos have a negative impact on ecosystem health through ARG dispersal. This research highlights a new threat for the health and conservation of the unique wildlife of the Galapagos, their ecosystems, and the humans inhabiting this World Heritage Site. Our recommendation to local policy makers is to control and reduce the use of antibiotics in both human and animal health, thus helping enforce antimicrobial regulations.

1. Introduction

Globally, we are experiencing a rapid escalation of antimicrobial resistance (AMR) combined with the equally rapid decline in discovery and development of new antibiotic classes (Bartlett et al., 2013). Infections caused by resistant bacteria are responsible for thousands of once preventable human deaths and the loss of millions of USD every year (O'Neil, 2014; WHO, 2020). The Pan American Health Organization (PAHO) has drawn the attention of the urgent need of strengthening antimicrobial surveillance in all different activities including veterinary practices (Acar and Moulin, 2013). A main challenge in veterinary medicine is the use of antimicrobials as growth promoters, prophylactics, and metaphylactics (Murphy et al., 2017). One Health has been internationally recognized as a collaborative effort of multiple disciplines to attain optimal health for people, animals, and the environment (Deem and Brenn-White, 2020). As such, the One Health paradigm addresses the negative impacts of AMR on the well-being of animals, humans, and the health of environments, together with the

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* Corresponding author. Charles Darwin Research Station, Charles Darwin Foundation, Santa Cruz, Galapagos, Ecuador.
E-mail addresses: ainoa.nieto@fcdarwin.org.ec (A. Nieto-Claudin), deem@stlzoo.org (S.L. Deem), rodfermc@vet.ucm.es (C. Rodríguez), scano@ucm.es (S. Cano), nicolas.moity@fcdarwin.org.ec (N. Moity), fredy.cabrera@fcdarwin.org.ec (F. Cabrera), esperon@inia.es (F. Esperón).

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need for transdisciplinary research and prevention plans to minimize this shared threat (Fisman and Laupland, 2010; Kahn, 2016; Amuasi et al., 2020).

Despite recent efforts to lessen this health threat, AMR is widely distributed across the world with resistant bacteria found in all continents and environments, both with and without evidence of significant human impacts (Segawa et al., 2013; Van Goethem et al., 2018). The development of resistance is an event that naturally occurs in microbial communities on a small scale (Tan et al., 2018); however, it is well-known that human activities play a key role in the dissemination of AMR throughout the environment (Carballo et al., 2016; Esperón et al., 2020). For instance, between 75 and 90% of the antibiotics administrated to livestock may be released into the environment through animal waste and widely dispersed through the use of slurry (Chee-Sanford et al., 2009; Wright, 2010). Antimicrobial resistance may be encoded by plasmid-mediated antimicrobial resistance genes (ARGs) that can be further disseminated among bacteria through horizontal gene transfer mechanisms via mobile genetic elements such as transposons, plasmids, and integrons (Wang et al., 2014). Therefore, ARGs are considered environmental pollutants of high concern for human, animal, and environmental health (Pruden et al., 2006).

As free-living wildlife generally do not receive antibiotics or veterinary care, the prevalence of AMR harbored by wildlife is generally accepted as influenced by the relative level of exposure to anthropogenic contamination (Ramey and Ahlstrom, 2020; Ahlstrom et al., 2021). As such, several studies confirm that wildlife may be good sentinels of the burden of resistance within the local environment and may therefore be useful for identifying potential point sources of anthropogenic AMR contamination (Blanco-Peña et al., 2017; Cevidanes et al., 2020; Sacristán et al., 2020; Ewbank et al., 2021).

Strict regulations and governmental operation plans have been delivered in several countries to reduce and control the dispersion of AMR (WHO, 2017). In Latin America, the Heads of Medicine Agencies (EAMI) have joined the battle against AMR in an effort to integrate policies and regulations that help to reduce it. Unfortunately, current national and international programs are generally biased toward human and domestic animal AMR bacterial surveillance, and the environment has only received limited attention (Dolejska and Literak, 2019). In 2019, Ecuador launched a strategic national plan to reduce AMR (Ecuadorian Ministry of Health, 2019); however, most antimicrobials for both human and animal use are still accessible throughout the country without a medical prescription.

The Galapagos Archipelago is located in the eastern tropical Pacific Ocean, almost 1000 km away from the coast of mainland Ecuador. Galapagos is a UNESCO World Heritage Site and is still considered one of the most well conserved and managed archipelagos on Earth; however, oceanic islands are highly sensitive to human impacts (Fordham and Brook, 2010; Veron et al., 2019). In recent years, anthropogenic environmental degradation has progressed rapidly in Galapagos due to land transformations through agriculture, urbanization, tourism, and the impacts of novel invasive species (Watson et al., 2009; Toral-Granda et al., 2017). The biggest terrestrial vertebrate inhabiting Galapagos are species of giant tortoises (*Chelonoidis* spp.), widely distributed across the archipelago in both natural and human-modified landscapes. From the 14 species of Galapagos tortoises that have ever been described, two are currently extinct and nine are considered endangered or critically endangered by the IUCN, 2020. Main threats to Galapagos tortoises include habitat loss and fragmentation, illegal trade, introduced and invasive species, global warming, egg loss to introduced predators, disease, and trauma (Blake et al., 2012; Ellis-Soto et al., 2017; Bastille-Rousseau et al., 2019; Frazier, 2021).

Preliminary results from our research group show that tortoises from the human-inhabited Santa Cruz Island carry ARGs (Nieto-Claudin et al., 2019) with 100% of the 28 samples positive to at least one gene conferring resistance. Based on these findings, we designed a broader study to better understand how ARGs are distributed across the archipelago and whether human activities such as farming and sewage may play a role on ARG abundance and distribution within inhabited islands. We hypothesized that tortoises inhabiting human-modified landscapes (Santa Cruz) would have a higher frequency and load of resistance gene-carrying bacteria than tortoises from non-inhabited areas (Alcedo Volcano, Isabela). Considering the fact that Santa Cruz tortoise species are migratory and move long distances every year from protected national park areas into urban and farming properties (Blake et al., 2013), our second hypothesis was that tortoises sampled across an anthropic gradient within the human-populated Santa Cruz Island would not present differences in frequency and ARG load since their movements suggest use of the landscape within these different categories of human land-use.

2. Materials and methods

2.1. Study site

We conducted the study on two islands of the Galapagos Archipelago (Fig. 1). Santa Cruz, located in the center of the archipelago and inhabited by humans, and the non-inhabited Alcedo Volcano, located in northern Isabela Island. Santa Cruz is the most populated island of Galapagos, with an estimated 15,700 inhabitants (National Institute of Statistics, 2015). In addition, the population of Santa Cruz has dramatically increased in the last few years because of tourism, with a record of 276,000 visitors arriving to Galapagos in 2018, and the majority of these visiting Santa Cruz, the most tourist trafficked island of the archipelago (Observatorio de Turismo de Galápagos, 2018). Isabela, by contrast, has a small population of less than 2500 inhabitants (National Institute of Statistics, 2015) with human settlements restricted to the southern part of the island (S00.975418°, W91.007818°).

Santa Cruz contains only two species of critically endangered giant tortoises, with the most predominant (*Chelonoidis porteri*) inhabiting the central and south-western area and the other (*Chelonoidis donfaustoi*) restricted to the north-eastern side of the island. For the current study we focus on *C. porteri* as the most representative and more widely distributed species of Santa Cruz. The estimated population for this species is 3400 individuals based on IUCN data from 2010 (Cayot et al., 2017), but no census has been conducted in the last decade. Through our work, we estimate a population that exceeds 6000 individuals (Blake, Cabrera, Nieto-Claudin, Deem unpublished data).

Alcedo Volcano is located in the middle of Isabela Island, south of the Equator (S00.4409454°, W091.1068907°) and is one of five volcanoes on Isabela inhabited by giant tortoises. As a result of isolation and evolution, each volcano has a unique species of giant tortoise, with *Chelonoidis vandenburghi* endemic to Alcedo and its slopes. The IUCN estimated population for this species is 6320 tortoises and its conservation status is vulnerable (Cayot et al., 2018). Alcedo Volcano has never been inhabited by humans; however, between 2004 and 2006 extensive goat eradication was carried out by dozens of hunters and rangers as part of the International Project Isabela (Lavoie et al., 2007). Since then, Alcedo Volcano has been restricted to scientific activities, with very few scientists visiting the caldera annually.

2.2. Sampling design and sample collection

We collected tortoise biological samples as part of a long-term health assessment within the Galapagos Tortoise Movement Ecology Programme (GTMEP). From 2017 to 2020, we collected samples from 200 free-living western Santa Cruz tortoises (including 28 samples from a pilot study, Nieto-Claudin et al., 2019) and 70 Alcedo Volcano tortoises, for several health research purposes. Samples in Santa Cruz were collected over multiple years and seasons across a gradient of human to non-human modified landscapes (i.e., farms, urban areas, and the national park). “Farm” and “urban” areas were considered the most human-modified, “touristic reserves” (private properties, with very few

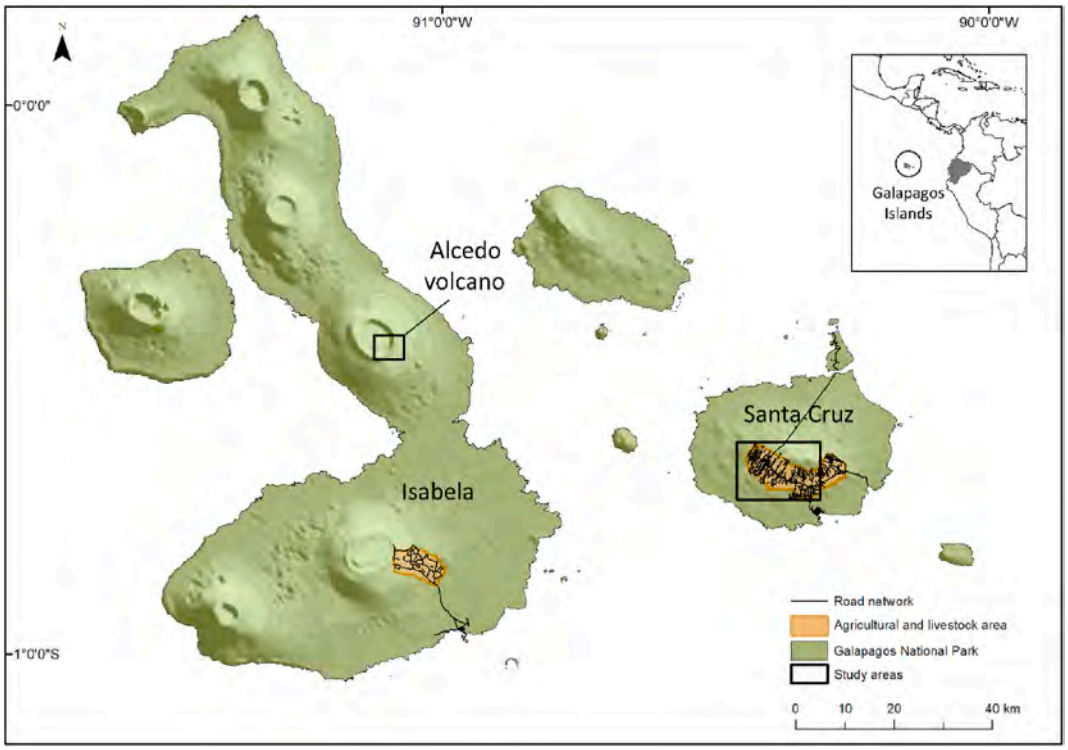

Fig. 1. Location of the Galapagos islands within the American continent (insert). Giant tortoise sampling areas to study ARGs within the Galapagos archipelago in relation to areas of human influence (agricultural and urban areas) on Santa Cruz and Isabela islands. Almost 97% of the archipelago is protected (Galapagos National Park).

human infrastructures, that focus on sustainable tourism where tortoises freely roam every year) were considered intermediate human impact, and "national park" was deemed the least human-impacted area since no people are allowed to conduct any activity other than research and targeted hunting of invasive species. Samples in Alcedo were collected during an 8-day field trip in July 2018 along the rim of the crater and the slopes of the volcano.

Due to handling challenges, we selected individuals weighing less than 200 kg. We recorded morphometric measurements and weighed each tortoise with a precision of ±0.5 kg. We performed a physical examination and determined the sex in mature animals by tail length and plastron concavity. We classified immature animals (sub-adults) based on curve carapace lenght (Nieto-Claudín et al., 2021). We collected feces from the cloaca and placed approximately 25 g per tortoise in a 15 ml sterile conical tube for ARG determination. We kept all samples frozen at −80 °C until analysis and for a maximum of 6 months. We identified tortoises by microchips previously placed by Galapagos National Park Service rangers. If no microchip was detected, we placed a new subcutaneous microchip (DATAMARS®) in the caudo-ventral area of the left hind leg to avoid sampling duplication.

We collected samples under the Galapagos National Park annual research permits PC-36-17, PC-35-18, PC-16-19, PC-28-20, and the International Animal Care and Use Committee from GREFA (Spain) with registration number 17/001. We stored all samples at the Charles Darwin Research Station (CDRS) and conducted the analyses at the INIA-CISA in Madrid, Spain. We transported frozen samples under exportation permits 153-2019-EXP-CM-FAU-DNB/MA, 192-2019-EXP-CM-FAU-DNB/MA, and 031-2020-EXP-CM-FAU-DNB/MA.

2.3. Molecular analysis of ARGs

We performed total DNA extraction directly from fecal samples by using a pressure filtration technique (QuickGene DNA Tissue Kit S, Fujifilm, Japan) following the manufacturer's instructions. The 16S rRNA gene was amplified in each DNA sample by quantitative PCR (qPCR) in 10-fold dilutions of extracted samples according to Jiang et al. (2013). A sample was considered as validated when a ten-fold dilution showed a cycle threshold (Ct) less than 25 (Esperón et al., 2018). Once validated, we analyzed samples by a panel of up to 21 different ARGs encoding resistance to eight different antimicrobial classes as representatives of the main antimicrobials generally used in veterinary and human medicine: tetracyclines (*tet*(A), *tet*(B), *tet*(Y), *tet*(K), *tet*(M), *tet*(Q), *tet*(S), and *tet*(W)), sulfonamides (*sul*I and *sul*II), aminoglycosides (*str* and *aad*A), phenicols (*cat*I and *cat*II), macrolides (*erm*B and *erm*F), quinolones (*qnr*S and *qnr*B), betalactams (*bla*TEM and *mec*A), and polymixins (*mcr*-1). We used the primers previously described in Nieto--Claudin et al. (2019). We quantified the 21 genes for each sample by the cycle threshold (Ct) for the 16S rRNA gene. We applied the following formula to estimate the percentage of bacteria harboring ARGs (load percentage of each ARG):

$$\%\ \mathrm{ARG} = 10^{[2+0.33(\mathrm{ct16S}-\mathrm{ctARG})]},$$

with results expressed in logarithm 10, ranging from −8 (given to a sample considered negative) to +2 (when 100% of the bacteria in the sample presented the ARG). The cycling parameters were the same for all qPCR reactions [6′ 95 °C, 40 × (10″ 95 °C, 30″ 60 °C)], with annealing and extension in the same step, at constant temperature of 60 °C. A melting curve step was performed at the end of the qPCR reaction (Nieto-Claudin et al., 2019). We classified a microbiome as multi-resistant when the fecal sample was positive to at least three genes encoding resistance to different classes of antimicrobials (Blanco-Peña et al., 2017).

2.4. Statistical analyses

We provided descriptive statistics for each gene within the total samples. We analyzed the normality of the data by Kolmogorov-Smirnov test ($p < 0.05$). We performed a non-parametric Mann-Whitney *U* test to assess differences between the two species (Alcedo Volcano and Santa Cruz), sex, age (adults and subadults), and sampling season (dry and

humid) due to violation of ANOVA model assumptions. We used Kruskal Wallis (K–W) with Bonferroni post-hoc adjustment to test for differences in the classes and numbers of ARGs between the four sampling areas of Santa Cruz.

To further explore the hypothesis that more human-modified areas would lead to higher loads of ARGs in giant tortoises, we ranked the sample areas on an anthropogenic gradient from least to most human impacts: Alcedo Volcano as the most preserved biotope, the national park area of Santa Cruz, touristic reserves of Santa Cruz, and urban and agricultural areas within Santa Cruz as the most human-modified biotopes. We used a logistic regression model to study the effect of anthropization on the presence of multi-resistant microbiomes and the presence of different antimicrobial classes, using Alcedo Volcano as the reference. We used a Poisson regression to model count data (total number of ARGs and antimicrobial classes per sample). We estimated Odds Ratio (OR) and 95% confident intervals (CI) for each variable. All analyses were performed on IBM® SPSS® Statistics 25.

To further explore spatial differences regarding ARGs within the Santa Cruz sampling areas, and to identify spatial clusters within those areas, we performed a hot spot analysis based on the Getis-Ord Gi* statistic (Getis and Ord, 1992) using ArcGIS 10.6.1 (ESRI, Redlands, CA). This statistic allows us to identify areas with individuals carrying significantly more ARGs in their microbiome than expected in a random distribution, given their distance and their value relative to the mean. Therefore, an individual with high ARGs should be surrounded by other individuals with high ARGs to constitute a significant hotspot. This statistic identifies spatial clustering by means of z-scores and assumes normality of data (Zhang, 2008). A high z-score value ($\geq$1.65) represents statistically significant clustering of high values (hotspot) at the p-value $\leq$ 0.1, a low negative value (<-1.65) represents spatial clustering of low values (cold spot), while a value close to zero indicates no spatial clustering.

3. Results

In Santa Cruz, 19 of the 21 ARGs were present in at least one sample. The most predominant genes were *tet*(W) and *tet*(Q) with 93% and 71% respectively, followed by *tet*(M) (35.5%), *tet*(S) (33.5%), *bla*TEM (33%), *qnr*S (26%), and *aad*A (25%). Genes *sul*I and *mcr*-1 were not detected, and the median genes per sample was 4 (min 0, max 10). By contrast, we found 18 of the 21 ARGs present in Alcedo Volcano. The most predominant genes were also *tet*(W) and *tet*(Q) with 51.4% and 50% respectively, followed by *bla*TEM (37.1%), *qnr*S (30%), and *tet*(B) (24.3%). Genes *tet*(Y), *tet*(M), and *erm*(B) were not detected, and the median genes per sample (2) was significantly lower than in Santa Cruz ($p < 0.001$). We found statistically significant differences among species with a higher number of tortoises carrying genes *tet*(M) ($p < 0.001$), *tet*(Q) ($p = 0.001$), *tet*(S) ($p < 0.001$), *tet*(W) ($p < 0.001$), *tet*(Y) ($p = 0.002$), *str* ($p = 0.026$), and *aad*A ($p < 0.001$) in Santa Cruz. The presence of *sul*I gene ($p = 0.017$) in Alcedo Volcano was also statistically significant. We did not find significant differences for any of the other genes. There were no differences based on sex or age.

Clustered by antimicrobial classes, the tortoise microbiome in both islands were observed to be characterized by the presence of tetracycline, beta-lactams, and quinolone resistant genes, in descending order of occurrence. However, in Santa Cruz the microbiome was also characterized by aminoglycoside resistance, having a statistically significant higher percentage of tetracyclines ($p < 0.001$) and aminoglycosides ($p < 0.001$) than in Alcedo Volcano (Fig. 2). The number of antimicrobial classes for which resistance genes were detected was also significantly different between the two tortoise species ($p = 0.003$).

Additionally, 68 of 200 Santa Cruz tortoises (34%) had a multi-resistant microbiome, whereas only 12 out of 70 Alcedo tortoises (17.1%) did. This difference was statistically significant ($p = 0.008$).

The quantification of genes showed significant differences for genes *tet*(M) ($p < 0.001$), *tet*(S) ($p < 0.001$), *tet*(W) ($p < 0.001$), *tet*(Y) ($p = 0.002$), *str* ($p = 0.027$), and *aad*A ($p < 0.001$), with a higher load of bacteria carrying these ARGs in Santa Cruz. Only the *sul*I gene showed a higher quantification in Alcedo ($p = 0.017$) (Table 1). The *mcr*-1 gene was only found in one individual from *C. vandenburghi* which was not statistically significant.

In Santa Cruz, and contrary to our second hypothesis, tortoises sampled within more anthropized areas (urban and farming locations) presented a significantly higher number of ARGs than those from less human-modified areas (touristic reserves and national park) ($p < 0.001$) (Fig. 3). Clustered by antimicrobial classes, farming areas showed a higher number of tortoises carrying tetracycline resistance than those from the national park ($p = 0.019$), and tortoises in urban areas had higher aminoglycosides than both the national park ($p = 0.009$) and touristic reserves ($p = 0.013$). Urban areas also presented a higher

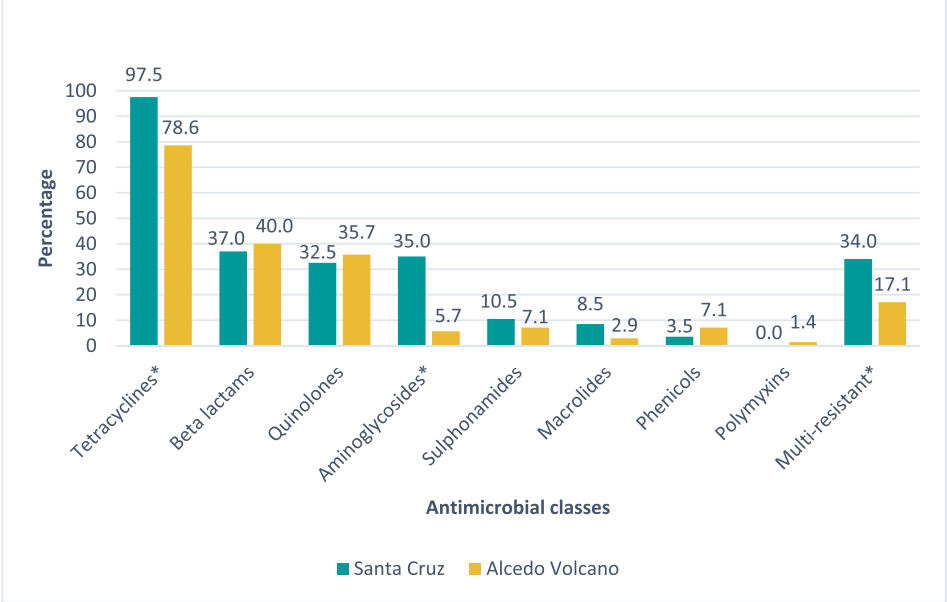

Fig. 2. Percentage of tested samples for Santa Cruz and Alcedo Volcano tortoise study areas that presented antimicrobial resistant genes categorized by antimicrobial classes. Multi-resistance is considered when more than two classes are present. (*) Statistically significant results.

Table 1
Quantification (log10 percentage of bacteria with each gene) of positive samples for each ARG (clustered by antimicrobial classes) for Santa Cruz and Alcedo Volcano tortoise study areas. Percentage (prevalence) - median (Quartile 1, Quartile 3) - and range (minimum, maximum).

Antimicrobial classes and genes	Santa Cruz tortoises (n = 200)			Alcedo Volcano tortoises (n = 70)		
	Prevalence %	Median (Q1, Q3)	Min- Max	Prevalence %	Median (Q1, Q3)	Min- Max
Tetracyclines	97.5			78.6		
tet(A)	14.5	−8.0 (−8.0, −8.0)	−8 to −2.8	7.1	−8.0 (−8.0, −8.0)	−8 to −2.4
tet(B)	17.5	−8.0 (−8.0, −8.0)	−8 to −2.1	24.3	−8.0 (−8.0, −7.3)	−8 to −0.5
tet (K)	6.5	−8.0 (−8.0, −8.0)	−8 to −3.1	2.8	−8.0 (−8.0, −8.0)	−8 to −2.7
tet (M)*	35.5	−8.0 (−8.0, −5.7)	−8 to −2.0	0		
tet (Q)	71	−4.6 (−8.0, −3.6)	−8 to −0.5	50	−6.6 (−8.0, −3.7)	−8 to −0.3
tet (S)*	33.5	−8.0 (−8.0, −5.5)	−8 to −0.7	7.1	−8.0 (−8.0, −8.0)	−8 to −2.1
tet (W)*	93	−3.9 (−4.7, −3.1)	−8 to −2.0	51.4	−5.5 (−8.0, −4.0)	−8 to −0.3
tet(Y)*	12	−8.0 (−8.0, −8.0)	−8 to −2.6	0		
Sulphonamides	10.5			7.1		
*sulI**	0			2.9	−8.0 (−8.0, −8.0)	−8 to −4.3
sulII	10.5	−8.0 (−8.0, −8.0)	−8 to −3.6	4.3	−8.0 (−8.0, −8.0)	−8 to 0.7
Aminoglycosides	35			5.7		
*str**	12	−8.0 (−8.0, −8.0)	−8 to −3.0	2.9	−8.0 (−8.0, −8.0)	−8 to −4.4
*aadA**	25	−8.0 (−8.0, −6.8)	−8 to −1.3	2.9	−8.0 (−8.0, −8.0)	−8 to −3.5
Phenicols	3.5			7.1		
catI	0.5	−8.0 (−8.0, −8.0)	−8 to-6.4	1.4	−8.0 (−8.0, −8.0)	−8 to −3.0
catII	3	−8.0 (−8.0, −8.0)	−8 to −2.7	7.1	−8.0 (−8.0, −8.0)	−8 to −2.0
Macrolides	8.5			2.9		
erm(B)	1.5	−8.0 (−8.0, −8.0)	−8 to −2.8	0		
erm(F)	7	−8.0 (−8.0, −8.0)	−8 to −1.5	2.3	−8.0 (−8.0, −8.0)	−8 to −4.5
Quinolones	32.5			35.7		
qnrB	9.5	−8.0 (−8.0, −6.0)	−8 to −2.8	11.4	−8.0 (−8.0, −4.2)	−8 to −1.1
qnrS	26	−8.0 (−8.0, −8.0)	−8 to −1.5	30	−8.0 (−8.0, −8.0)	−8 to −3.1
Beta-lactams	37			40		
blaTEM	33	−8.0 (−8.0, −5.0)	−8 to −0.6	37.1	−8.0 (−8.0, −4.1)	−8 to −0.7
mecA	5	−8.0 (−8.0, −8.0)	−8 to −3.3	4.3	−8.0 (−8.0, −8.0)	−8 to −3.4
Polymyxins	0			1.4		
mcr-1	0			1.4	−8.0 (−8.0, −8.0)	−8 to −3.9

*Statistically significant.

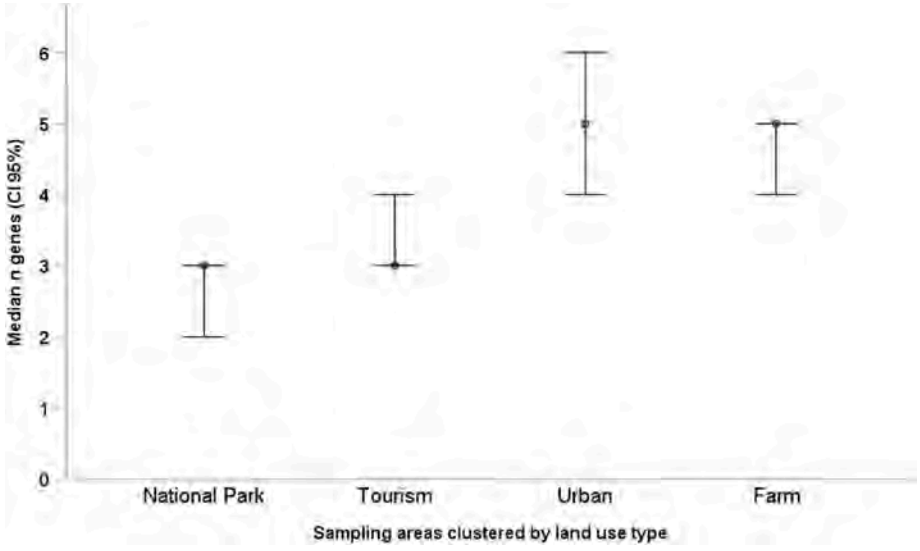

Fig. 3. Graphic representation of median and 95% CI for the number of ARGs presented in giant tortoises (*C. porteri*) from different sampling areas of Santa Cruz Island, clustered by land use type.

number of tortoises carrying macrolide resistance than touristic reserves, but Bonferroni post-hoc adjustment was approaching significance ($p = 0.07$).

We found a positive correlation between human activities (i.e., farming, agriculture, deforestation, sewage, infrastructure, domestic animals) and the presence of AMR. Logistic regression predicted a higher risk of finding multi-resistant microbiomes for touristic areas and urban and agricultural areas when compared to Alcedo Volcano, with an increment of 2.5- and 3-fold respectively. Regarding antimicrobial classes, the probability of finding resistant microbiomes to aminoglycosides was also strongly associated with anthropization, having a probability 5.8-, 6.5-, and 12.2-fold increase for park, touristic, and urban and agricultural zones, respectively (Table 2).

In Santa Cruz, Poisson regression predicted a risk of having more ARGs per sample of 2.3-fold in the national park area and 2.4-fold in touristic reserves. Interestingly, the risk of having higher ARGs increased 11.2-times in urban and agricultural areas of Santa Cruz when compared to the isolated Alcedo Volcano. The risk of having multi-drug resistance increased by 1.8-fold in urban and agricultural zones when compared to the Volcano (Table 3).

Table 2
Logistic regression model of multi-resistant microbiomes and aminoglycoside resistant genes on a gradient of human-modified sampling areas, in relation to Alcedo Volcano. Different areas correspond to Santa Cruz national park areas, Santa Cruz touristic reserves, and Santa Cruz urban and farming areas.

Variables	Sampling areas	p value	OR Exp (ß)	95% C.I.for OR [Exp(ß)]	
				Lower	Upper
Multi-resistance	Constant	<0.001	0.207	0.111	0.385
	National Park	0.373	1.519	0.606	3.810
	Touristic Reserves*	0.034	2.486	1.071	5.771
	Urban and Agriculture*	0.003	3.040	1.451	6.369
Aminoglycosides	Constant	<0.001	0.061	0.022	0.166
	National Park*	0.004	5.824	1.746	19.429
	Touristic Reserves*	0.002	6.513	2.016	21.047
	Urban and Agriculture*	<0.001	12.233	4.139	36.151

*Statistically significant.

Table 3
Poisson regression model of antimicrobial classes and ARGs on a gradient of human-modified sampling areas, in relation to Alcedo Volcano. Different areas correspond to Santa Cruz national park areas, Santa Cruz touristic reserves, and Santa Cruz urban and farming areas.

Variables	Sampling areas	p value	OR Exp (ß)	95% C.I.for OR [Exp(ß)]	
				Lower	Upper
Number of ARGs	Constant	<0.001	12.358	8.524	17.917
	National Park*	0.01	2.352	1.231	4.495
	Touristic Reserves*	0.005	2.416	1.300	4.489
	Urban and Agriculture*	<0.001	11.206	6.334	19.825
Number of classes	Constant	<0.001	5.964	4.361	8.156
	National Park	0.37	1.266	0.755	2.122
	Touristic Reserves	0.09	1.554	0.934	2.586
	Urban and Agriculture*	0.01	1.77	1.147	2.729

*Statistically significant.

Results from the hot spot analysis identified two main areas in Santa Cruz where sampled tortoises with higher numbers of ARGs clustered spatially with a 90–99% confidence ($p < 0.1$ and $p < 0.01$). These areas correspond to a) a peripheral urban zone that has recently been converted into an industrial area; and b) cattle farms near Occidente and El Carmen districts in the highlands of Santa Cruz (Fig. 4a and b respectively). Cold spots from this analysis (i.e., areas with tortoise clusters that have significantly lower ARGs) all correspond to touristic and national park areas where anthropogenic activities are absent or very reduced (Fig. 4c).

4. Discussion

We found ARGs to be present in both Santa Cruz (*C. porteri*) and Alcedo Volcano giant tortoises; however, a significantly higher prevalence of tetracyclines, aminoglycosides, number of ARGs, number of antimicrobial classes, and prevalence of multi-drug resistance was found in tortoises on the human-inhabited island of Santa Cruz when compared to the Volcano. Moreover, statistical analyses comparing Santa Cruz sampling areas support a positive correlation between human activities and the level of ARGs in free-living giant tortoises. Both farming and urban areas showed a higher prevalence of AMR when compared to the less human-modified areas of touristic reserves and national park. The hot spot analysis performed for Santa Cruz Island showed a positive correlation between the higher number of ARG and the presence of human activities (e.g., sewage, cattle industry, human settlements), where all hot spots were found either within farming properties or within highly impacted human areas and former landfills. These hot spot areas all correspond with tortoises sampled near main roads, feeding on plastic and human waste contaminated landscapes, and freely roaming and feeding at cowsheds. By contrast, cold spots from the same analyses were found only within touristic reserves and national park areas where no pollution is found, and no farming activities can be performed.

Despite the fact that Santa Cruz tortoises are partially migrants and move long distances from dry protected areas into humid anthropized areas every year (Blake et al., 2013), a recent study shows how the use of agricultural land is segregated by body size, sex, and philopatry, with tortoises using an average of four farms a year and presenting a strong philopatry for the same areas every season (Pike et al., 2021). According to Pike et al. (2021), some individuals tend to remain in private land for up to six months a year, which means that they may be exposed to, and feed on, resistant-contaminated grass and water sources for a long period of time every year, supporting our findings in relation to higher number of ARGs and multi-resistance in more human-modified areas.

The prevalence of multi-resistance microbiomes in Santa Cruz free-living tortoises (34%) is noteworthy when compared to other studies based on the same methodology in wildlife species from remote archipelagos. In Ewbank et al. (2021), an AMR study on seabirds comparing biotopes showed a prevalence of 11.1% multi-drug resistance in the human-inhabited Fernando de Noronha Archipelago (Brazil). By contrast, Sacristán et al. (2020) found a 43% multi-resistance prevalence in wild felids ($n = 51$) from mainland Chile, with a positive association of anthropization landscapes and multi-resistance microbiomes. Considering these results, multi-resistance prevalence for Santa Cruz giant tortoises appears to be intermediary as compared to foxes inhabiting highly modified environments in Chile and seabirds inhabiting a remote but developed Brazilian archipelago. Differences among the published studies could be attributable to different factors: the consumption of antimicrobials for human, veterinary and agricultural use, or the microbial communities of the different host species as well as geographical, environmental, and climatic factors, which could favor the persistence of antimicrobial compounds and resistant bacteria. In the case of pristine or less-anthropized areas, differences among studies could also be due to the composition and diversity of plant and fungal communities, as well as soil bacteria. It is known that soil bacteria are one of the most natural sources for ARGs development, which is highly influenced by the type of soil (Álvarez-Martínez et al., 2020; Wang et al., 2020). We cannot reject that other species such as migratory birds may play a role in the dissemination of ARGs in the Galapagos Islands as it has been suggested in other environments, including genes of public health interest such as *mcr*-1 and β-lactamase genes (Hernández and González-Acuña, 2016; Cao et al., 2020). Therefore, to investigate the contribution of human activities on the presence of ARGs in the environment, it is necessary to carry out studies comparing more anthropized scenarios with less anthropized ones, using model species.

Based on our results, the microbiome of tortoises inhabiting the human-populated island of Santa Cruz is characterized by tetracycline and aminoglycoside resistant genes. The predominance of genes codifying for tetracycline resistance is in agreement with all other studies conducted in wildlife species with the same methodology (Cevidanes et al., 2020; Sacristán et al., 2020; Ewbank et al., 2021).

In Santa Cruz, some of the selected antibiotics used in veterinary medicine (mainly for free-ranging cattle farming, swine, and small animals) include penicillin, oxytetracycline, gentamicin, erythromycin, and streptomycin based on local interviews conducted from our research team. This information is in agreement with some of our findings on genes *tet*(M), *tet*(S), *tet*(W), *tet*(Y), str, *aad*A, and *erm*(F) found to be higher in human-modified landscapes (Santa Cruz) than in the isolated

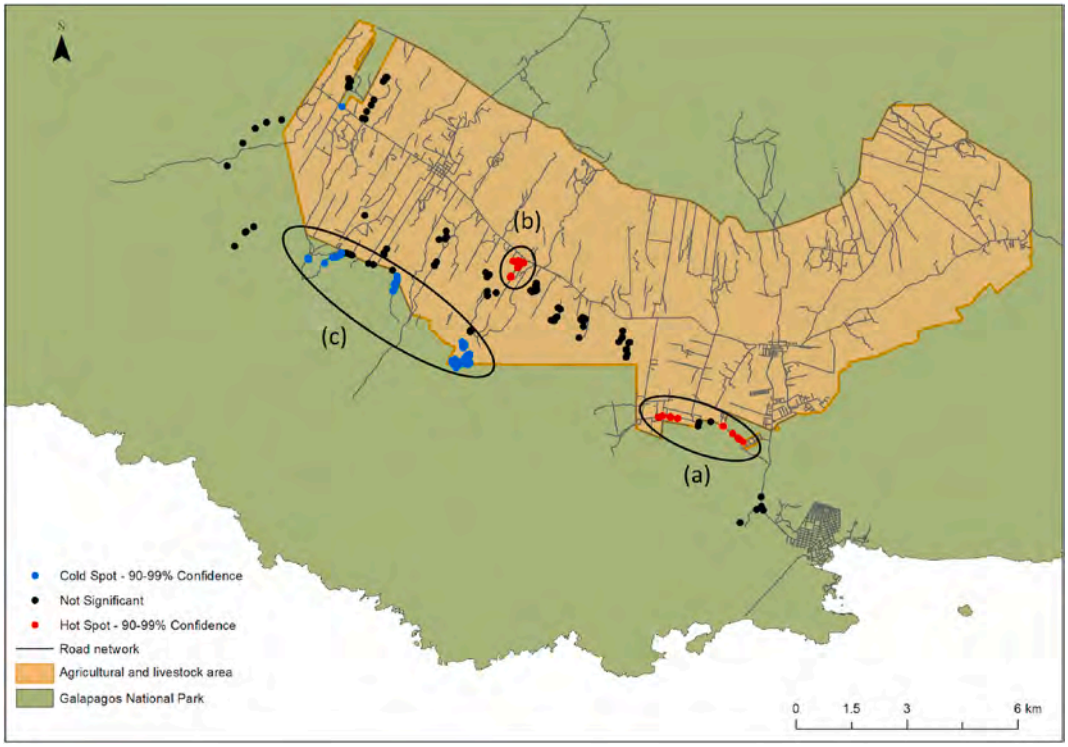

Fig. 4. ARGs cluster analysis within the Santa Cruz study area. Dots represent: hot spots in red (i.e., cluster areas with significantly more tortoises with a high number of ARGs), cold spots in blue (i.e., cluster areas with significantly more tortoises with a low number of ARGs), and non-significant clusters in black. (a) Hot spot cluster in a peripheral urban zone converted into an industrial area; (b) hot spot in cattle farms; (c) cold spots in touristic and national park areas. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Alcedo Volcano. Moreover, the risk of finding resistant bacteria to aminoglycosides increased meaningfully with human activities such urbanization and farming. Aminoglycosides are broad-spectrum antibiotics ranked by WHO (WHO, 2019) as critically important antimicrobials for human medicine and often is used to treat nosocomial and zoonotic infections (e.g., plague, tularemia, leishmania) as well as used as active components of topical medication (Krause et al., 2016; Van Duijkeren et al., 2019). In veterinary practice, aminoglycosides are broadly used to treat a wide range of pathologies including gastrointestinal, respiratory, and genitourinary infections, sepsis, and in-feed prophylaxis and metaphylaxis for chickens and swine. A recent survey of antimicrobial use in animals, published in 2016 by the World Organization for Animal Health (OIE, 2016), found that antimicrobial growth promoters were authorized for use in 80% of American countries that participated in the survey (in contrast with 40% in Asia, 18% in Africa, and 3% in Europe). In the Americas, the main food-producing animal species reported were cattle, poultry, and pigs. There are no official reports of the use of human or animal antibiotics in Ecuador, but most antimicrobial drugs can be acquired with no prescription.

The prevalence of antibiotic-resistant bacteria among wildlife occurs to be dependent on a variety of factors, such as habitat use and foraging strategy of the species sampled, particularly as they relate to anthropogenic inputs into the environment. Foraging represents a mechanism by which wildlife may be exposed to anthropogenic AMR contamination in the environment and could therefore be useful to identify those more exposed species that could be used as sentinels of the environmental resistome (Ramey and Ahlstrom, 2020). Furthermore, a positive correlation between the presence of microplastics and AMR have been found (Pham et al., 2021) and can therefore explain the higher prevalence of ARGs found in tortoises feeding on high polluted environments, and frequently observed eating plastics and garbage (Nieto-Claudin, pers. obs.). As such, we propose that giant tortoises may serve as good sentinels of Galapagos ecosystem health since they are widespread, inhabiting both human and non-human populated islands, and are long-lived species that commonly feed within cattle and farming areas and near human settlements of Santa Cruz. As we demonstrate in this study, levels of ARGs and multi-resistance in tortoises are directly correlated to level of anthropic impacts on landscapes.

Several efforts have been conducted recently in Galapagos to enhance the importance of integrating scientific outputs into local and regional policies and management decisions. A research agenda was created in 2017 as a result of a multi-institutional and multi-sectorial exercise (Izurieta et al., 2018), and three main issues for Galapagos sustainability were identified: human-population growth, climate change, and invasive species. Moreover, new approaches have been proposed to address potential conservation conflicts between local farmers and tortoises (Benitez-Capistros et al., 2019) and to redesign the livestock production systems in the archipelago, with the aim to achieve socio-ecological sustainability (Puente-Rodríguez et al., 2019). Unfortunately, to date human and animal health have not been given the attention and importance they require to ensure a sustainable and equitable development of the archipelago.

In conclusion, our results support the hypothesis that human activities in Galapagos are driving the dispersion of antibiotic resistant bacteria into the environment with potential consequences for human and animal health. This study advances the detection and evaluation of ARGs in Galapagos endemic species and adds to the growing body of knowledge on human-related impacts on the biodiversity and ecosystems of the Galapagos Islands. We recommend our results to be used for informing local policies that reinforce the proper use, control, and outreach on antibiotics for both human and veterinary medicine, together with a redesign of waste management practices leading to more sustainable and responsible stewardship. Moreover, this research highlights a new threat for wildlife, human, and ecosystem health that may threaten the conservation of the Galapagos archipelago and the well-being of human inhabitants. These potential threats from the spread of

AMR include water and land pollution, the transmission of resistant bacteria to other wildlife species, domestic animals, and human beings, compromising medical treatment success in both human and animal diseases, and negative impacts on normal microbiome and by extension to the normal immune system function of animals and humans. Further studies should focus on identifying the specific sources and pathways that lead ARGs to pollute the environment and for proposing alternative and creative solutions to limit and control antibiotic use within this World Heritage Site. We recommend implementing long-term monitoring of giant tortoise health and AMR prevalence across the Galapagos Islands to better understand the One Health implications of AMR and how wildlife might act as reservoirs and spreaders of ARGs that may threaten animal and human health and wellbeing.

Credit author statement

Ainoa Nieto Claudin: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Project administration, Funding acquisition. Sharon L. Deem: Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. Casilda Rodríguez: Formal analysis, Writing – review & editing, Supervision. Santiago Cano: Formal analysis, Writing – review & editing. Nicolas Moity: Formal analysis, Writing – review & editing. Freddy Cabrera: Methodology, Investigation. Fernando Esperón: Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision.

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.

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