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Antimicrobial resistance genes present in the faecal microbiota of free-living Galapagos tortoises (*Chelonoidis porteri*)

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Abstract

Antimicrobial resistance (AMR), encoded by plasmid-mediated AMR genes (ARGs), is an increasing global public health threat. Wildlife play a fundamental role as sentinels, reservoirs and potential vectors of ARGs. For the first time in Galapagos, we have identified and quantified the presence of ARGs in free-living giant tortoises (*Chelonoidis porteri*). We performed ARG analyses by quantitative PCR of faeces collected from the cloaca of 30 tortoises widely distributed across Santa Cruz Island. Validated samples (n = 28) were analysed by a panel of up to 21 different ARGs and all 28 tortoise samples were positive to one or more genes encoding resistance. Thirteen of 21 tested ARGs were present in at least one sample, and 10 tortoises (35.7%) had a multi-resistant pattern. We recommend additional research so we may more fully understand resistance patterns across taxa and geographical locations throughout the Galapagos archipelago, and the implications of ARGs for the health of wildlife, domestic animals, and humans. In this study, we found 100% of sampled giant tortoises had ARGs present in their faeces, suggesting a large-scale distribution of these genes within the archipelago.

KEYWORDS
antibiotic resistance, Galapagos Islands, giant tortoises, multi-resistant patterns, wildlife surveillance

1 | INTRODUCTION

Antimicrobial resistance (AMR) presents an increasing global public health threat that involves all major microbial pathogens and antimicrobial drugs (Greger, 2007; Levy & Marshall, 2004; Marinho, Santos, Gonçalves, Poeta, & Igrejas, 2016). The main cause of AMR is the misuse of antibiotics in veterinary and human medicine since antibiotics are not only prescribed for treatment, but are also administered as disease prevention measures and growth promotion (Guerra, Fischer, & Helmuth, 2014). In developing countries, antimicrobial drugs are readily available in community pharmacies and improper use also contributes to the emergence of AMR (Sakeena, Bennett, & McLachlan, 2018).

Free-living wildlife play a fundamental role as sentinels, reservoirs and potential vectors of AMR (Blanco-Peña et al., 2017; Jobbins & Alexander, 2015; Pruden, Pei, Storteboom, & Carlson, 2006). AMR has entered wildlife populations around the world and has in some places been shown to be more frequent among wildlife than in human or livestock populations in the same regions (Vittecoq et al., 2016). Since AMR is greater when antibiotic exposure increases (e.g., due to anthropogenic use) (van de Sande-Bruinsma et al., 2008), the diversity and abundance of AMR in wild animals may be used as an indicator of human activities (e.g., over-use in livestock), at the interface between wildlife, domestic animals and humans.

Most studies on AMR surveillance in wildlife are performed by culture-dependent methods, using a limited number of bacterial

strains presented in the microbiota (e.g., *Escherichia coli*) as indicators of AMR. However, since most bacteria are not cultivable (Eckburg et al., 2010), and the most relevant resistance determinants (i.e., AMR genes [ARGs]) are codified into mobile genetic elements, mainly plasmids which are not captured in culture methods, the detection of AMR using culture-dependent methods might not be representative of the whole ARG microbiota. Consequently, recent studies emphasize the advantage of characterizing the environmental spread of AMR by using non-culture-dependent methods directly from the microbiome (Esperón, Sacristán, Carballo, & Torre, 2018; Jiang et al., 2013; Wang et al., 2014).

As the human footprint expands into the last wilderness areas on earth (Di Marco, Venter, Possingham, & Watson, 2018), the potential for AMR impact on previously isolated wildlife populations is increasing with consequences largely unknown. The endemic biotas of oceanic islands are highly vulnerable to human impacts. On the Galapagos Islands, human colonization occurred relatively recently and the archipelago retains most of its original endemic biodiversity (Causton et al., 2013). Nevertheless, anthropogenic environmental degradation has progressed rapidly due to recent land transformations through agriculture, urbanization, tourism, and the impacts of novel invasive species (Toral-Granda et al., 2017).

Antimicrobial resistance has been identified as a potential threat to the unique fauna of the Galapagos; however, previous studies have been performed only by culture methods, and samples were collected far from human-inhabited areas, showing inconclusive results (Thaller et al., 2010; Wheeler, Hong, Bedon, & Mackie, 2012). Farm activities in the Galapagos often cause ecological and sanitary impacts on natural ecosystems and may introduce unknown quantities of antibiotics under poorly controlled conditions.

Critically endangered Galapagos tortoises (*Chelonoidis* spp.) are emblematic animals with the very name "Galapagos" stemming from the Spanish word for tortoise. Threatened by centuries of over-harvesting, they remain endangered due to habitat and climate change, invasive species and other human impacts (Deem, Cruz, Higashiguchi, & Parker, 2012; Deem, Cruz, et al., 2008; Gibbs, Snell, & Causton, 1999; Watson et al., 2010). Characterized as generalist herbivores, Galapagos tortoises undertake migrations driven by spatiotemporal variability in vegetation productivity (Blake, Guézou, Deem, Yackulic, & Cabrera, 2015; Yackulic, Blake, & Bastille-Rousseau, 2017). These giants move long distances, from protected national park areas to human-modified landscapes, including tourist farms and areas managed for livestock on human-inhabited islands such as Santa Cruz (Blake et al., 2015, 2012). Despite the identification of human–animal cohabitation as one of the most critical factors for the conservation of wildlife species (Daszak, Cunningham, & Hyatt, 2001; Deem, Parker, & Miller, 2008), few studies have been performed to assess how this cohabitation may affect tortoise health and the spread of AMR in the archipelago.

Here, we respond to this deficit by identifying, quantifying and reporting, for the first time in Galapagos, on the presence of ARGs in the symbiotic gut bacteria among free-living giant tortoises (*Chelonoidis porteri*) on Santa Cruz Island.

Impacts

- Antimicrobial resistance has become one of the main public health threats worldwide.
- We found antibiotic resistance genes in 100% of free-living giant tortoises sampled.
- Direct detection of antimicrobial resistance genes offers a more sensitive technique for environmental studies when compared to microbiological culture.

2 | MATERIALS AND METHODS

2.1 | Study site

We conducted the study on Santa Cruz, a 986 km^2 island that rises to a maximum elevation of 860 m. The Galapagos National Park covers almost 90% of Santa Cruz with native vegetation dominate at low and middle elevations, but introduced plant species are throughout the central highlands due to farming and tourism on privately owned land (114 km^2). Santa Cruz is the most populated island in the archipelago with an estimated 15,700 inhabitants (National Institute of Statistics and Census, 2015). Most people are concentrated in a small town (Puerto Ayora) on the southern coast of the island, with smaller rural settlements along roads throughout the south-central region of the island.

2.2 | Sampling design and sample collection

In October 2017, we collected samples from 30 free-roaming tortoises from different locations along their seasonal migration routes (Bastille-Rousseau et al., 2016), including humid and transitional habitats throughout the National Park (protected areas), rural (agricultural, livestock), and urban zones (within Puerto Ayora town limits; Figure 1).

To facilitate animal handling, we selected individuals weighing <200 kg. We collected faeces from the cloaca and placed approximately 25 g per tortoise in a 15-ml sterile Falcon tubes for the ARG studies. We kept all samples frozen at −20°C until analyses. We identified tortoises by microchips previously placed by Galapagos National Park Service rangers. Tortoises that had not been previously pit tagged were given a subcutaneous microchip (DATAMARS®) placed in the caudoventral area of the right hind leg. We also collected a number of other biomaterials (e.g., blood, swabs, carapace scrapes) and recorded morphometric measurements as part of an ongoing health assessment of the Galapagos tortoises across the islands. We collected samples under the Galapagos National Park research permit PC-36-17 and the International Animal Care and Use Committee from GREFA, Spain, with registration number 17/001.

2.3 | Molecular analysis of ARGs

We performed the analysis of ARGs by quantitative PCR (qPCR) directly from faecal samples. Following faecal thaws, we carried out

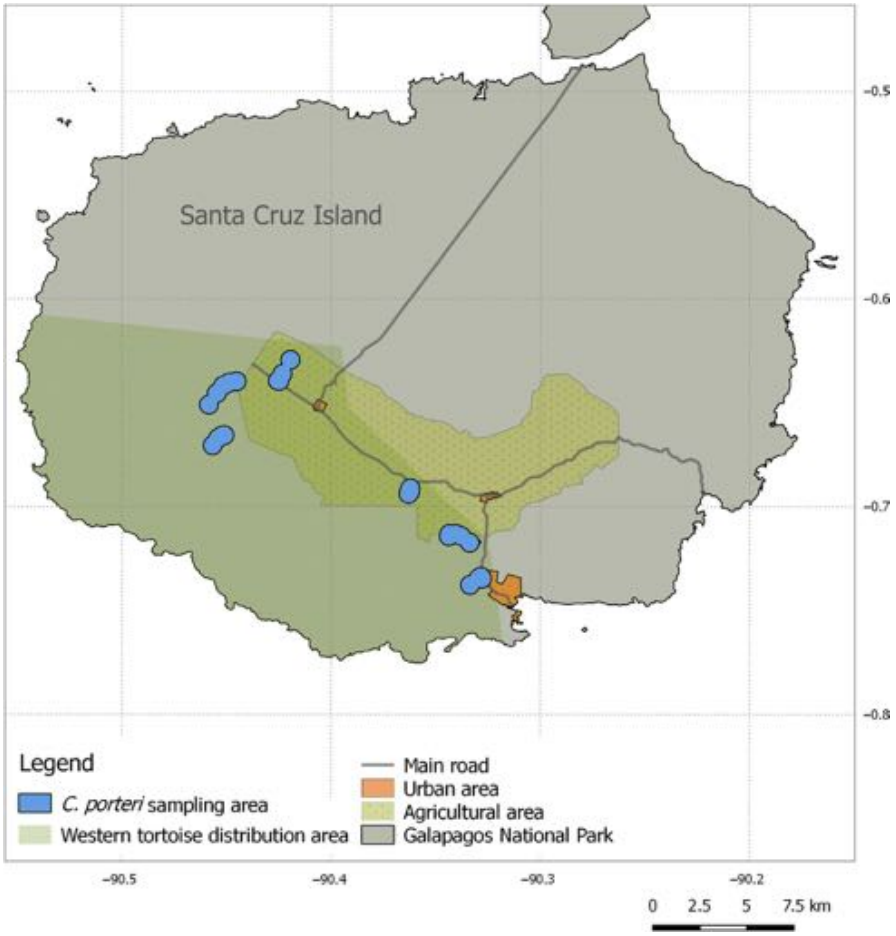

FIGURE 1 Galapagos tortoise (*Chelonoidis porteri*) sampling areas for study of ARGs in relation to areas of human influence on Santa Cruz Island (agricultural and urban areas). Notice almost 90% of the island is protected (Galapagos National Park)

DNA extraction by a pressure filtration technique (QuickGene DNA tissue kit S, Fujifilm®) under the manufacturer's instructions. We validated DNA extraction through the detection of the 16S rRNA gene by qPCR based on SYBR Green® (Jiang et al., 2013). Once validated, we analysed samples by a panel of up to 21 different ARGs. We chose representative ARGs of the main antimicrobial classes used in veterinary and human medicine (i.e., tetracyclines, sulphonamides, phenicols, macrolides, aminoglycosides, beta-lactams, quinolones and polymyxins), with many of them of clinical relevance (Table 1). In the case of mcr-1 gene, we designed a set of primers (see Table 1), based on the sequences available in the GenBank. This qPCR was validated with an *E. coli* isolate previously tested as positive to mcr-1, kindly provided by VISAVET Research Group (Faculty of Veterinary Medicine, Universidad Complutense de Madrid, Spain). We then tested field samples of different origins (faeces from different species, soil and manure) with this qPCR and sequenced any positive amplifications in order to evaluate whether unspecific amplification was observed. We performed sensitivity of the test with 10-fold dilutions of a cloned PCR product for a positive detection up to 8.4 copies per reaction. For all genes, we added PCR-positive controls and both DNA extraction and PCR-negative controls. We quantified the 21 genes for each sample by the cycle threshold (ct) for the 16S rRNA gene. We developed a custom-made formula as follows: $\log_{10}$ (percentage of an ARG) = $2+0.33*(ct_{16SrRNA} - ct_{ARG})$, where ct is the cycle threshold (16S rRNA is for the bacterial determination and ARG is for each gene), and the value 0.33 is the mean slope for all the genes tested. We expressed results in the $\log_{10}$ of the hypothetical percentage of bacteria that each gene presents, for the percentage load of ARG. Our custom-made formula also allows the results to be expressed by percentage, and it is highly correlated with those previously published by Xie et al. (2016) (R^2 = .997; data not shown).

2.4 | Statistical analyses

We obtained qualitative results for both single ARGs and antimicrobial classes, and considered the microbiome potentially resistant to an antimicrobial class if the sample was positive for at least one gene within a class. We calculated the percentage of "multi-resistant microbiomes," as previously described by Blanco-Peña et al. (2017). We applied the term "multi-resistant microbiome" when a faecal sample was positive to at least three genes encoding resistance to different classes of antimicrobials. We also obtained quantitative results for each ARG within each sample. For statistical analysis, we defined a negative sample as any value ≤−8 since we calculated the sensitivity of the technique using a baseline threshold of −7 (i.e., 0.0000001% of bacteria with one ARG). We performed descriptive statistics (minimum, maximum, and average) for each gene within the total

TABLE 1 Antimicrobial resistance genes selected for the Santa Cruz tortoise (*Chelonoidis porteri*) study clustered by antimicrobial classes, their mechanism of analysis and references

Resistance to	Gene	Mechanism	Reference
Aminoglycosides	*aad*A	Deactivation	Devarajan et al. (2016)
	Str	Deactivation	Wang et al. (2014)
β-lactams	bla_{TEM}	Deactivation	Devarajan et al. (2016)
	*mec*A	Ribosomal protection	Francois et al. (2003)
Macrolides	*erm(B)*	Ribosomal protection	Chen, Yu, Michel, Wittum, and Morrison (2007)
	erm(F)	Ribosomal protection	Chen et al. (2007)
Phenicols	*cat*I	Deactivation	Jiang et al. (2013)
	*cat*II	Deactivation	Jiang et al. (2013)
Polymyxins	*mcr*-1	Deactivation	Current study[a]
Quinolones	*qnr*B	Ribosomal protection	Cummings et al. (2011)
	*qnr*S	Ribosomal protection	Marti & Balcazar (2013)
Sulphonamides	*sul*I	Ribosomal protection	Jiang et al. (2013)
	*sul*II	Ribosomal protection	Jiang et al. (2013)
Tetracyclines	*tet*(A)	Efflux pump	Jiang et al. (2013)
	tet(B)	Efflux pump	Jiang et al. (2013)
	tet(K)	Efflux pump	Jiang et al. (2013)
	tet(M)	Ribosomal protection	Jiang et al. (2013)
	tet(Q)	Ribosomal protection	Jiang et al. (2013)
	tet(S)	Ribosomal protection	Jiang et al. (2013)
	tet(W)	Ribosomal protection	Jiang et al. (2013)
	tet(Y)	Efflux pump	Jiang et al. (2013)

[a]Primers for *mcr*-1 are as follows: *mcr*-1F: 5′-TGATACGACCATGCTCCAAA-3′; mcr-1R: 5′-GCCACCACAGGCAGTAAAAT-3′.

samples. Additionally, we represent ARG quantification results by hierarchical clustering using a heat map.

3 | RESULTS

Thirteen of the 21 ARGs (61.9%) for which we tested were present in at least one sample. Genes *tet*(Q) and *tet*(W) were the most common (100% and 96.4%, respectively), followed by *aad*A (39.3%), bla_{TEM} (32.1%), *qnr*B (28.6%), *erm*(F) (17.8%), *str* (14.A3%), *tet*(B) (10.7%), *sul*II (7.1%) and 3.6% for genes *tet*(A), *tet*(M), *mec*A and *qnr*S. Genes *tet*(Y), *tet*(K), *tet*(S), *sul*I, *cat*I, *cat*II, *erm*(B), and *mcr*-1 were not detected. The average number of ARGs per sample was 3.6 (mode of three, with a minimum of two and a maximum of six genes).

In the 28 samples that passed validation and clustered by antimicrobial classes, 100% were positive to one or more genes encoding resistance for tetracycline, 42.9% for aminoglycosides, 32.1% for beta-lactams, 28.6% for quinolones, 17.9% for macrolides, and 7.1% for sulphonamides. None of the tortoise faecal samples contained ARGs encoding phenicol or polymyxin resistance (Figure 2). The average number of antimicrobial classes that were presented in each sample was 2.3, and 80% of the samples presented ARGs for two or more antimicrobial classes.

Additionally, 10 of 28 tortoises (35.7%) had microbiomes with a multi-resistant pattern (Table 2). We detected several patterns of multi-resistance. The most frequent patterns included the combination of tetracyclines with aminoglycosides and tetracyclines with beta-lactams. The combination of beta-lactams, tetracyclines, and aminoglycosides was associated with 5 of 10 microbiomes (50.0%), whereas sulphonamides were only present in 2 of 10 multi-resistant patterns (20.0%). Macrolides and quinolones were associated with 4 of 10 multi-resistant patterns (40.0%) but never presented in the same pattern together. One of the 10 patterns (10.0%) contained ARGs from five different antimicrobial classes (i.e., tetracyclines, sulphonamides, aminoglycosides, quinolones, and beta-lactams).

Results of ARG quantification were represented with a heat map (Figure 3), showing values below the 1% of bacteria harbouring each gene ($\log_{10}$ values ranging −7 to −0.5). The highest level was a sample with 0.29% of bacteria harbouring the *tet*(Q) gene, with the other 27 samples with ARG quantification below 0.01% (Table 3).

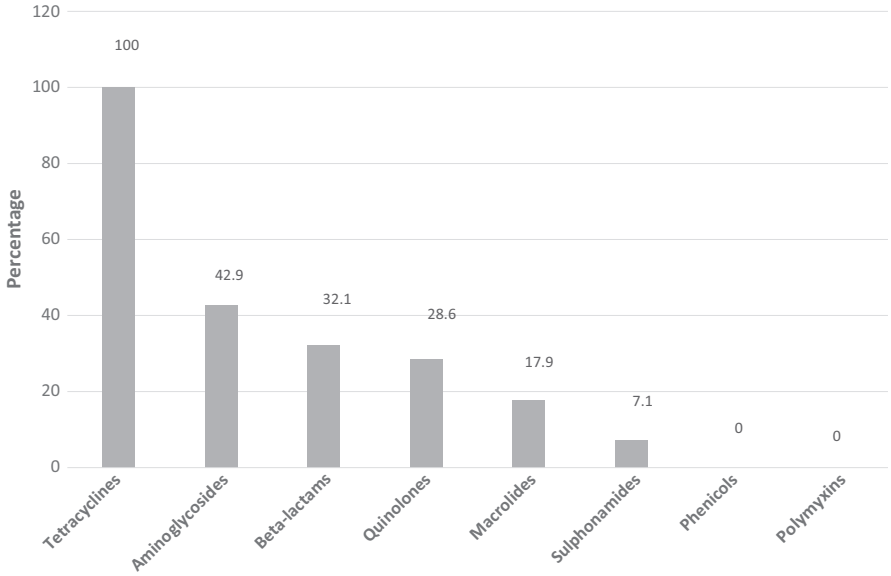

FIGURE 2 Percentage of tested samples for Santa Cruz tortoise (*Chelonoidis porteri*) study that presented antimicrobial resistance genes categorized by antimicrobial classes

TABLE 2 Patterns of multi-resistant genes for the Santa Cruz tortoise (*Chelonoidis porteri*) study

Id number	Pattern[a]	*n* genes	Genes detected
689173	tet-amin-blac-quin-sul	6	*tet*(Q), *tet*(W), *sul*II, *str*, *qnr*B, bla_{TEM}
702961	tet-amin-blac-quin	5	*tet*(Q), *tet*(W), *aad*A, qnrB, bla_{TEM}
697079	tet-amin-blac-quin	5	tet(Q), *tet*(W), *aad*A, qnrB, qnrS, bla_{TEM}
694751	tet-amin-blac	4	*tet*(Q), *tet*(W), *aad*A, mecA
681540	tet-amin-blac	4	*tet*(Q), *tet*(W), *aad*A, bla_{TEM}
674304	tet-blac-quin	4	*tet*(Q), *tet*(W), *qnr*B, bla_{TEM}
685390	tet-amin-macr	6	*tet*(A), *tet*(B), *tet*(Q), *tet*(W), *aad*A, *erm*(F)
683519	tet-amin-macr	5	*tet*(Q), *tet*(W), *str*, *aad*A, *erm*(F)
628743	tet-blac-macr	4	tet(Q), *tet*(W), *erm*(F), bla_{TEM}
690633	tet-macr-sul	5	tet(B), *tet*(Q), *tet*(W), *sul*II, *erm*(F), bla_{TEM}

[a]tet: tetracycline; amin: aminoglycoside, blac: beta-lactam; quin: quinolone; sul: sulphonamide; macr: macrolide.

4 | DISCUSSION

All 28 Galapagos tortoise faecal samples we tested were positive to one or more genes encoding antibiotic resistance. This prevalence is high in comparison with previous results from Galapagos, although previous studies used culture methodologies and only included animals living away from high human-impacted areas (Thaller et al., 2010; Wheeler et al., 2012). Results by culture methods reported the presence of AMR "virtually absent" from land iguanas, with ARGs only detected in two of 96 samples (Thaller et al., 2010). In addition, Wheeler et al. (2012) reported the presence of resistant bacteria in reptiles from tourism sites on Plaza Sur and San Cristobal Islands, but only genes *tet*(A) and *tet*(B) were described from *E. coli* cultures, with an estimated prevalence of 30% (18 of 59 tested samples). Our results are higher than these two previous studies, which may be a function of our focus on a species that travels between high and low human-impacted areas, and/or the utilization of a more sensitive molecular diagnostic.

In the present study, the average number of ARGs detected per sample was 3.6. This value is higher when compared to the 2.2 average value in the only other known study of ARGs in wildlife in which qPCR was used on pigeon faecal samples (Blanco-Peña et al., 2017). This

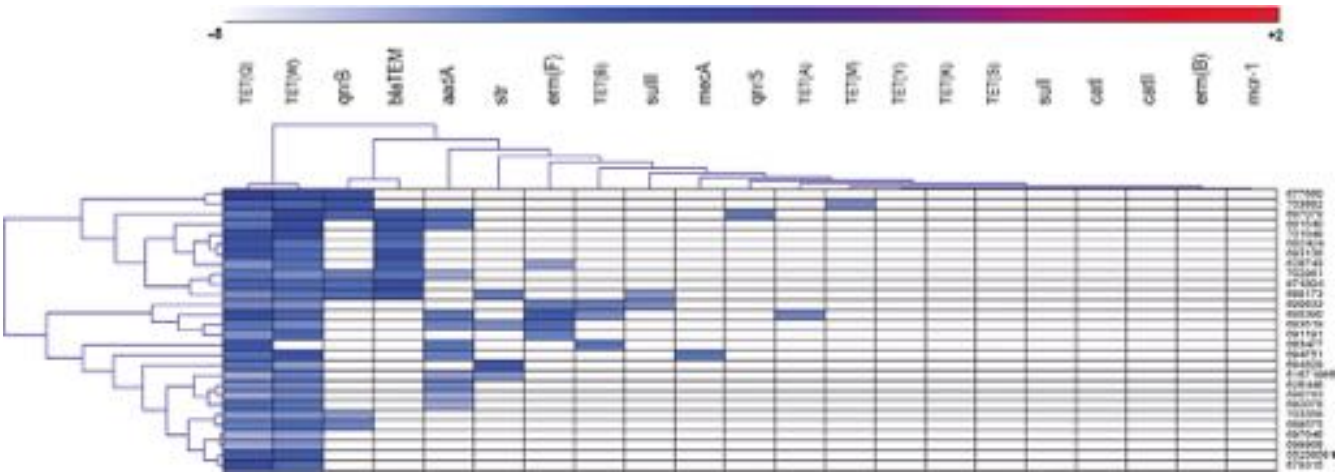

FIGURE 3 Hierarchical clustering of samples and genes from Santa Cruz tortoise (*Chelonoidis porteri*) study, with a heat map. White cells correspond with negative results, whereas blue to red gradient represents values from 0.0000001% to 100% of bacteria with ARG. The 21 genes that were tested are located in the headline, and microchip identification numbers of study individuals are located along the right-hand side

TABLE 3 Quantification (log_{10} percentage of bacteria with each gene)—mean (minimum/maximum)—and percentage of positive samples for each ARG for Santa Cruz tortoise (*Chelonoidis porteri*) study, clustered by antimicrobial classes

Antimicrobial families	Genes	Quantification (Log10) Average (min/max)	% Positive results (*n* = 28)
Tetracyclines	*tet*(A)	−7.9 (−8.0/−4.5)	3.6
	tet(B)	−7.6 (−8.0/−3.9)	10.7
	tet(Y)	−8.0 (−8.0/−8.0)	0
	tet(K)	−8.0 (−8.0/−8.0)	0
	tet(M)	−7.9 (−8.0/−4.7)	3.6
	tet(Q)	−3.9 (−5.8/−0.5)	100
	tet(S)	−8.0 (−8.0/−8.0)	0
	tet(W)	−4.0 (−8.0/−2.1)	96.4
Sulphonamides	*sul*I	−8.0 (−8.0/−8.0)	0
	*sul*II	−7.8 (−8.0/−4.8)	7.1
Aminoglycosides	*str*	−7.5 (−8.0/−3.0)	14.3
	*aad*A	−6.7 (−8.0/−3.8)	39.3
Phenicols	*cat*I	−8.0 (−8.0/−8.0)	0
	*cat*II	−8.0 (−8.0/−8.0)	0
Macrolides	*erm*(B)	−8.0 (−8.0/−8.0)	0
	erm(F)	−7.3 (−8.0/−3.1)	17.8
Quinolones	*qnr*(B)	−6.8 (−8.0/−2.5)	28.6
	qnr(S)	−7.3 (−8.0/−3.1)	3.6
Beta-lactams	*bla*TEM	−6.8 (−8.0/−2.2)	32.1
	*mec*A	−7.9 (−8.0/−3.9)	3.6
Polymyxins	*mrc*-1	−8.0 (−8.0/−8.0)	0

result suggests how farm areas may show higher contamination of AMR than urban areas (where pigeons were sampled), indicating how important it is to develop antibiotic good practices within the farming industry.

Genes *qnr*B, *qnr*S and bla_{TEM}, all associated with farming activities (Dohmen et al., 2017; Esperón et al., 2018; Jones-Dias, Manageiro, & Caniça, 2016) and widely distributed in South America and Ecuador (Armas-Freire et al., 2015; Salles, Zurita, Mejía, Villegas; Latin America Working Group on Bacterial Resistance, 2013), were found in faeces of eight, one and nine tortoises, respectively. This finding is noteworthy since quinolones and beta-lactams are broad-spectrum antibiotics commonly used to treat human infections, and the appearance of resistances may compromise the treatment of bacterial infectious diseases in both humans and animals.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is widely distributed throughout the world, including mainland Ecuador (Planet et al., 2015). Originally, healthcare-associated MRSA infections have emerged in the community and also from livestock in recent years (Stefani et al., 2012), becoming epidemic and causing severe disease and often fatal outcomes in humans (DeLeo & Chambers, 2009). MRSA is listed as "High Priority" by the World Health Organization in their global priority list of antibiotic-resistant bacteria to guide research, discovery and development of new antibiotics (Tacconelli & Magrini, 2017). This is the first report of the *mec*A gene in Galapagos; importantly, this discovery was in the faeces of a giant tortoise that freely moves across the most human-populated island.

Regarding multi-resistant microbiomes, and in contrast with Blanco-Peña et al. (2017), sulphonamides were not the most predominant genes associated with multi-resistant patterns, being found in only two samples. The combination of beta-lactams, tetracyclines, aminoglycosides and/or quinolones was associated with 6 of 10 microbiomes (60%). The low prevalence of *sul* genes found in this study agrees with previous results based on culture methods in Galapagos, in which only one out 23 isolates showed resistance to trimethoprim–sulphamethoxazole (Wheeler et al., 2012).

Unlike iguanas and many other wildlife species in the Galapagos, giant tortoises commonly overlap with farmlands, increasing the risk of ingestion and dissemination of AMR throughout the environment. Due to their long migrations, tortoises are potential vectors for the spread of ARGs throughout the biotic community, and may act as key indicators of environmental health. Tracking the health status of tortoises may allow for a better understanding of threats that not only affect the conservation of these tortoises, but also the health of the entire ecosystem. Lastly, we recommend the proper use of antibiotics in veterinary and human medical practices in the Galapagos. If implemented, this would greatly minimize exposure to ARGs for humans, domestic animals, and wildlife. Antibiotic best practices and local outreach and advice must be a priority to minimize the presence and impacts of antibiotic resistance in the iconic wildlife species and unique ecosystems of the archipelago.

To the authors' knowledge, this is the first time that qPCR has been used for the detection and quantification of ARGs in wildlife species in the Galapagos. These data contribute to the conservation of one of the most iconic animals of the islands and in turn may help direct management decisions for the well-being of Galapagos' human and non-human inhabitants alike.

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CONFLICT OF INTEREST

Ainoa Nieto Claudin, Fernando Esperon, Stephen Blake and Sharon L. Deem declare there is no conflict of interest.

We followed all institutional and national guidelines for the care and use of animals. Samples were collected under the Galapagos National Park research permit PC-36-17 and the International Animal Care and Use Committee from GREFA, Spain, with registration number 17/001. Samples were transported following all national and international guidelines, under the Galapagos National Park authorization permit 102-2017 and the Galapagos Biosecurity Agency authorization permit 235-ABG-2017.

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