American Journal of Animal and Veterinary Sciences

Original Research Paper

Tetracycline Resistance Profile in Darwin Finches in the Galapagos Islands

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Article history
Received: 16-07-2022
Revised: 19-10-2022
Accepted: 20-10-2022

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Abstract: Antimicrobial Resistance (AMR), which is the ability of microorganisms to withstand attack by antimicrobial drugs, has a worldwide impact. Thus, it is important to identify resistance mechanisms circulating in the environment. Among antimicrobials widely used in human and veterinary health, tetracycline is ideal due to its safety and has been categorized by the WHO as a critically important antimicrobial. Based on the fact that wild animals are bioindicators of environmental contamination and that wild birds are considered sentinels of AMR in the ecosystem, in the present study, we studied AMR patterns in Darwin finches (*Geospiza* spp.) in the Galapagos Islands, Ecuador. To this end, a total of 384 cloacal swabs from Darwin finches were collected from three zones of Santa Cruz Island (an urban, an agricultural, and a protected zone). Phenotypic antibiotic resistance was analyzed by the Kirby-Bauer disk diffusion method in *Escherichia coli* (n = 136) and *Enterococcus* spp. (n = 332) isolates. PCR was performed for the detection of the *tet*A gene in *E. coli* strains and of the *tet*M gene in *Enterococcus* isolates. Resistance for at least one of nine and one of eight antimicrobials was observed in 62.5% (85/136) and 28.6% (95/332) of *E. coli* and *Enterococcus* isolates, respectively. A high percentage of phenotypic resistance to tetracycline was identified for both bacteria. The *tet*A and *tet*M genes were identified in 83.7% (36/43) of *E. coli* strains and 75.4% (49/65) of *Enterococcus* spp. isolates, respectively. The highest percentages of AMR were observed in the agricultural zone. We also found the presence of multi-drug-resistant strains. These results show that Darwin finches might be proposed as sentinels of AMR in the Galapagos Islands.

Keywords: Antimicrobial Resistance, Tetracycline, Darwin Finches, Galapagos, Multi-Resistance

Introduction

Antimicrobial Resistance (AMR), which is the ability of microorganisms to withstand attack by antimicrobial drugs, is a global health problem related to several factors, including bacterial genetics and the human, veterinary and agricultural usage of antibiotics (Marston *et al*., 2016). Among important antimicrobials, tetracycline, which is a broad-spectrum antimicrobial that acts against Gram-positive and Gram-negative bacteria (Marosevic *et al*., 2017), has been categorized as highly critical (WHO, 2018). Since this microbial is considered ideal due to its safety (Grossman, 2016), it is widely used in human and veterinary medicinal practices (Thaker *et al*., 2010). However, some bacteria can develop resistance against tetracycline. The main mechanisms of tetracycline resistance are the activation of efflux pumps and ribosomal protection (Rossolini *et al*., 2017). Efflux

pumps are codified by 23 different genes, among which *tet*A is the most common one, whereas ribosomal protection involves 11 genes, among which *tet*M is frequently detected (Sigirci *et al*., 2019).

Studies in wildlife have allowed the understanding of the role of mobile genetic elements in AMR dissemination (Alonso *et al*., 2021) and identifying those related to AMR. In particular, wild birds have been proposed as sentinels of AMR dissemination in diverse environments (Bonnedahl and Järhult, 2014). In this context, isolated environments such as the Galapagos Islands, which are located approximately 1000 Km from Ecuador's continental coast (UNESCO, 2022), can be useful to evaluate the anthropogenic impact on the spread of AMR (Nieto-Claudin *et al*., 2021). Among the passerine endemic birds that inhabit the Galapagos Islands, Darwin finches have been widely studied as a model of adaptive radiation (Hau and Wikelski, 2001), which is a phenomenon related to the fact that these birds adjust the morphology of their beaks in association with their nutritional habits (Michel *et al*., 2018). Eighteen species of finches, most of which belong to the group of ground or tree finches (*Geospiza* spp.), have been described on the islands (Hau and Wikelski, 2001). Ground finches feed especially on seeds but can be opportunistic in terms of their diet (Knutie *et al*., 2019).

To identify AMR patterns circulating in the Galapagos Islands, this study aimed to analyze sentinel AMR strains of *Escherichia coli* and *Enterococcus* spp. isolated from Darwin finches in three zones of Santa Cruz Island with different anthropogenic impacts: An urban, an agricultural, and a protected zone.

Materials and Methods

Sample Collection

A total of 384 Darwin ground finches (*Geospiza* spp.), were caught using mist nets from the urban (n = 128), agricultural (n = 128), and protected zones (n = 128), between March and August 2019. Cloacal swabs were collected from each bird. All cloacal swab samples were placed in 700 µL of brain heart infusion broth (BHI broth BBL™) at 37°C for 24 h in the laboratory of the Agency for the Regulation and Control of Biosafety and Quarantine for the Galapagos (ABG) at Santa Cruz Island. Samples were preserved in BHI with 20% of glycerol and transported to the laboratory of the School of Veterinary Medicine of the Universidad Central del Ecuador in Quito city, Ecuador, for further analysis.

Isolation and Identification of Enterococcus spp. and Escherichia Coli

For isolation of *Enterococcus* spp., samples were incubated in 10 mL BHI medium at 37°C for 24 h and one loopful was streaked in BBL CHRO Magar Orientation agar (Becton Dickinson, Heidelberg, Germany). For *E. coli*, one loopful was streaked in Levine eosine methylene blue agar (Becton Dickinson, Heidelberg, Germany). At least one colony phenotypically compatible with *Enterococcus* spp. was picked from the medium and isolated for further biochemical identification with bile esculin (Becton Dickinson, USA), pyrrolidinyl arylamidase (PYR-A-ENT, Britania, CABA, Argentina), leucine aminopeptidase (Britania, CABA Argentina) and sodium chloride 6.5% assays (Lopardo, 2016). At least one colony compatible with *E. coli* in Levine agar was identified using a biochemical battery (Triple Sugar, Iron Agar (BBL), Sulfide Indole Motility medium (BBL), Simmons Citrate Agar (BBL), Lysine Iron Agar (BBL) and urease broth (BBL). Identification of *Enterococcus* and *E. coli* isolates was further confirmed by MALDI TOF MS analysis.

Antibiotic Resistance Analysis

Phenotypic antibiotic resistance was analyzed by the Kirby-Bauer disk diffusion method (Hudzicki, 2009). For *Enterococcus* spp., eight classes of antibiotics were included: Ampicillin (10 µg), gentamicin (120 µg), chloramphenicol (30 µg), tetracycline (30 µg), vancomycin (30 µg), streptomycin (300 µg), teicoplanin (30 µg) and ciprofloxacin (5 µg), whereas for *E. coli* the antibiotics tested were: Ampicillin (10 µg), ciprofloxacin (5 µg), cefoxitine (30 µg), cefotaxime (30 µg), cefepime (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg).

The results were interpreted following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2019). Tetracycline phenotypic resistant isolates were further considered for *tet* gene analyses.

DNA Extraction and PCR for the Detection of the tetA and tetM Genes

DNA from the *Enterococcus* spp. and *E. coli* isolates was extracted by the boiling method (Millar *et al*., 2000). Both *tet*A and *tet*M PCR reactions included nuclease-free water and a final concentration of Buffer 1X, 0.2 mm dNTPs, 1.5 mm $MgCl_2$, and 0.025 U/µL Taq polymerase (GoTaq®DNA Polymerase, Promega). All primers were used at a concentration of 0.1 pM/µL.

Specific primers for *tet*A were tetA-F 5´-TTTCGGCGAGGATCGCTTTCACTG-3´ and tetA-R 5´-ATCCACCTGCCTGGACAACAT TGC -3´ (product size of 283 bp), whereas for the identification of the *tet*M gene, the primer sequences were TETM3 5´-GAACTCGAACAAGAGGAAAGC -3´ and 5´-ATGGAAGCCCAGAAAGGAT-3´ (amplicon length of 740 bp) (Cochetti *et al*., 2005). PCR conditions were the ones described elsewhere (Cochetti *et al*., 2005; Pantozzi, 2018). Amplicons were visualized by electrophoresis in 1.5% agarose gel and stained with SYBR safe (Invitrogen™).

Statistical Analysis

The association between AMR and the zone of sample collection was analyzed by the Chi-square test for differences among proportions, considering a p-value (<0.05) with a 95% confidence interval.

Results

Isolation and Identification of Enterococcus spp. and Escherichia Coli

A total of 136 *E. coli* and 332 *Enterococcus* spp. isolates were recovered from Darwin finches. Isolate distribution was associated with the sampling zone (p<0.0001). *E. coli* was isolated in a higher percentage in the agricultural zone at 47% (64/136), followed by the protected zone at 34% (46/136) and the urban zone at 19% (26/136). On the other hand, *Enterococcus* spp. was isolated more often in the urban zone with 41% (135/332), followed by the agricultural zone with 34% (114/332) and the protected zone with 25% (83/332).

AMR Profiles in E. Coli and Enterococcus spp. Isolates

AMR in *E. coli* was tested for nine antimicrobials, whereas that in *Enterococcus* spp. isolates were tested for eight antimicrobials (WHO/AGISAR, 2017). Resistance to at least one antimicrobial was observed in 62.5% (85/136) of *E. coli* strains. Resistance to ampicillin was identified in 50% of the isolates, to tetracycline in 31.6%, trimethoprim/sulfamethoxazole in 18.4%, chloramphenicol in 14.7%, to cefoxitin in 11.1%, to ciprofloxacin in 6.6%, to cefotaxime in 0.7% and cefepime in 0.7%. No resistance was identified for gentamicin. A total of 51 (37.5%) *E. coli* strains were susceptible to all the antimicrobials tested. Results showed no significant association between ampicillin resistance and the sampling zone. Differently, resistance to tetracycline, which was the second antimicrobial with a higher percentage, was significantly observed in the agricultural zone of the island (p<0.0001). *E. coli* isolates showed 23 resistance patterns, presenting resistance from one to eight antimicrobials (Table 1). Resistance pattern 21 (30.6%) was the most frequent one, followed by patterns 22 (10.6%), 15 (9.4%), and 7 (7.1%). Also, 26 *E. coli* isolates showed a Multi-Resistant (MDR) pattern.

For *Enterococcus* spp., resistance to at least one antimicrobial was recorded in 28.6% (n = 95) of the isolates. High resistance rates to tetracycline (19.6%) were found, followed by streptomycin (6.9%) and ciprofloxacin (5.7%). Lower AMR resistance rates were recorded for vancomycin (2.4%), ampicillin, chloramphenicol (1.8%), gentamicin, and teicoplanin (1.2%). Tetracycline resistance was observed in a higher proportion in the agricultural zone (p<0.0001). *Enterococcus* spp. isolates showed 14 resistance patterns, presenting resistance from one to four antimicrobials (Table 2). Resistance pattern 12 was the most frequent one (43.2%), followed by patterns 14 (9.5%), 8 (10.5%), and 13 (9.5%). Moreover, seven *Enterococcus* spp. isolates presented MDR phenotypes.

Table 1: Antimicrobial resistance patterns in *E. coli*

Pattern	Resistance Pattern	N° of Antimicrobials	N° of *E. coli* strains
1	A+F+C+FE+T+SXT+CI+CL	8	1
2	A+F+T+SXT+CL	5	1
3	A+F+T+SXT+CI	5	1
4	A+T+SXT+CI+CL	5	1
5	A+F+T+CL	4	2
6	A+T+SXT+CIP	4	3
7	A+T+SXT+CL	4	6
8	A+T+CIP+CL	4	1
9	T+SXT+CI+CL	4	1
10	A+F+T	3	2
11	A+T+SXT	3	2
12	A+T+CL	3	1
13	A+SXT+CI	3	1
14	T+SXT+CL	3	3
15	A+F	2	8
16	A+T	2	7
17	A+SXT	2	3
18	A+CI	2	1
19	A+CL	2	1
20	T+SXT	2	2
21	A	1	26
22	T	1	9
23	CI	1	2

A: Ampicillin; F: Cefoxitin; C: Cefotaxime; Fe: Cefepime; T: Tetracycline; SXT: Trimethoprim/sulfamethoxazole; CI: Ciprofloxacin; CL: Chloramphenicol

Table 2: Antimicrobial resistance patterns in *Enterococcus* spp.

Pattern	Resistance pattern	N° of Antimicrobials	N° of *Enterococcus* spp. strains
1	A+T+VA+TC	4	3
2	T+S+CI	3	2
3	CL+T+CI	3	1
4	CL+T+S	3	1
5	CL+T	2	4
6	S+CI	2	1
7	T+CI	2	3
8	T+S	2	10
9	VA+TC	2	1
10	A	1	3
11	CN	1	4
12	T	1	41
13	S	1	9
14	CI	1	12

A: Ampicillin; T: Tetracycline; VA: Vancomycin; TC: Teicoplanin; S: Streptomycin; CI: Ciprofloxacin; CL: Chloramphenicol

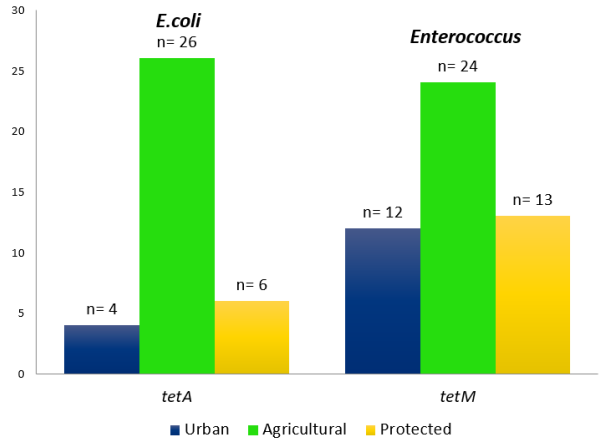

Fig. 1: Identification of the *tet*A gene in *E. coli* and the *tet*M gene in *Enterococcus* spp. in isolates with phenotypic tetracycline resistance, collected from Santa Cruz Island.

Detection of Tetracycline Resistance Genes

The *tet*A gene was identified in 83.7% (n = 36) of the *E. coli* isolates phenotypically resistant to tetracycline. The proportion of *tet*A gene-positive isolates was higher in the agricultural zone ($p<0.0001$) than in the urban and protected zones (Fig. 1).

The *tet*M gene was detected in 75.4% (n = 49) of the *Enterococcus* spp. strains phenotypically resistant to tetracycline. The proportion of *tet*M gene-positive isolates was higher in the agricultural zone ($p<0.05$) than in the urban and protected zones (Fig. 1).

Discussion

AMR is a multifactorial problem affecting both human and animal health (White and Hughes, 2019). In this context, environmental studies of AMR are important to understand AMR mechanisms circulating in diverse ecological niches. Wild species are good sentinels of AMR in the environment (Ramey and Ahlstrom, 2020) and, particularly, wild birds are important reservoirs and spreaders of AMR genes (Santos *et al*., 2013; Bonnedahl and Järhult, 2014; Foti *et al*., 2017).

In the present study, we identified tetracycline as an antimicrobial with a very high percentage of resistance in both *E. coli* and *Enterococcus* spp. isolates. Previous studies have shown that AMR could be associated with the use of antimicrobials in agricultural practices (Blanco *et al*., 2007). The use of antibiotics is known to exert selection pressure, favoring AMR in these microbial communities (Wall *et al*., 2016). Tetracycline is a broad-spectrum antibiotic used in both humans and animals (Thaker *et al*., 2010). Due to its good safety and tolerability, this antimicrobial has been used widely, a fact reflected in the resistance pattern observed in clinical and veterinary medicine (Grossman, 2016).

In addition, the presence and dissemination of AMR genes in the environment have been associated with the use of manure as fertilizer in agriculture (Lima *et al*., 2020). Since tetracycline is excreted as an active compound, the presence of *tet* genes increases after manure is used as a fertilizer (Guo *et al*., 2018; Xiong *et al*., 2018).

In the present study, we found tetracycline resistance in 31.6% of *E. coli* strains. High tetracycline resistance profiles in *E. coli* isolated from wild birds have also been reported by Guenther *et al*. (2010) in Germany, where 46.6% (n = 7/15) of the strains were resistant to this antimicrobial. Also, Giacopello *et al*. (2016); Nowaczek *et al*. (2021) have reported 48.2% (40/83) and 50% (n = 16/32) of tetracycline resistance respectively, in diverse wild birds from Europe. Similarly, Wheeler *et al*. (2012) reported resistance to tetracycline as the most frequent one in reptiles in the Galapagos Islands. In the present study, resistance to tetracycline was higher in the agricultural zone of the island, which may be related to its use in farming practices in the zone. It has to be considered that tetracycline is an antibiotic commonly used in livestock and poultry production (Michalova and Schlegelova, 2004).

Antimicrobials are usually absorbed in low amounts in the intestinal tracts of animals (Sarmah *et al*., 2006) and are consequently excreted in the environment without being degraded (Liu *et al*., 2020). Therefore, manure use in agriculture has been associated with the presence and dissemination of AMR genes in the ecosystem (Lima *et al*., 2020). Livestock fecal matter may act as a deposit of AMR genes in the soil, promoting resistance to clinically important antimicrobials (Lee *et al*., 2017). As tetracycline is excreted as an active compound, the presence of *tet* genes increases after manure is used as a fertilizer (Guo *et al*., 2018; Xiong *et al*., 2018).

Another important result of the present study was that *Enterococcus* spp. isolates were mostly non-susceptible (71.4%) to any of the antimicrobials tested in this investigation. Remarkably, in those resistant strains, high resistance rates were observed for tetracycline (19.6%). This result is similar to the one reported by Radimersky *et al*. (2010), who identified 20% of resistance for this antimicrobial in *Enterococcus* spp. strains isolated from feral pigeons in the Czech Republic. Also, Klibi *et al*. (2015) reported 19.2% of resistance to tetracycline in *Enterococcus* spp. isolated from wild birds in Tunisia.

In the present study, 19.1% of *E. coli* isolates presented an MDR profile. This result is in agreement with those of a study on seagulls in Alaska, where 22% of *E. coli* isolates were MDR (Atterby *et al*., 2016). Also, in Italy, Gambino *et al*. (2021) reported that 23% of *E. coli* strains from wild birds were MDR.

As in this investigation, other studies have reported *Enterococcus* spp. MDR strains were isolated from wild birds (Silva *et al*., 2018; Stępień-Pyśniak *et al*., 2019). Although the percentage was low (2.1%), these observations show that wild birds may be reservoirs of MDR bacterial strains, a fact that might be related to an anthropogenic impact mediated by contact with human or agricultural wastes (Skarżyńska *et al*., 2021).

E. coli strains with a phenotypic tetracycline resistance pattern were analyzed for the *tet*A gene. In general, tetracycline resistance in the family *Enterobacteriaceae* is given by the acquisition of efflux pump-coding genes, particularly *tet*A, which has been reported in other studies (Sigirci *et al*., 2019; Handrova and Kmet, 2019). In the present study, we identified this gene in 83.7% of *E. coli* strains isolated from Darwin finches. In agreement with this, Santos *et al*. (2013) identified the *tet*A gene in 60% (3/5) of tetracycline phenotypic resistant *E. coli* isolates in wild birds from the Azores archipelago. Also, Radhouani *et al*. (2012) reported the *tet*A gene in 59.3% (n = 16/27) of common buzzards in Portugal. The high proportion of this gene could be influenced by the transference of genetic resistance determinants through mobile elements such as conjugative transposons (Schell, 2019).

In the present study, we also determined the *tet*M gene in 75.4% of *Enterococcus* spp. strains from finches. The *tet*M gene is associated with ribosomal protection and it is usually identified in these bacteria (Frazzon *et al*., 2010). A high percentage of this gene has been reported in *Enterococcus* spp. strains isolated from wild birds (Radimersky *et al*., 2010; Santos *et al*., 2013; Yahia *et al*., 2018; Stępień-Pyśniak *et al*., 2019). In agreement with our results, in fecal samples from giant tortoises (*Chelonoidis* spp.) from Santa Cruz Island, determined 97.2% of *tet* genes, 35.5% of which corresponded to *tet*M genes. Nevertheless, these researchers did not associate the gene with the bacterial genus.

AMR in Galapagos finches may be related to an anthropogenic impact especially related to agricultural practices on the island. Wild birds act as reservoirs and disseminators of AMR. This research shows their importance to better understanding resistance mechanisms circulating in this specific niche, as well as to gain insights into the impact of human practices in the Galapagos Islands.

Conclusion

To our knowledge, this is the first AMR study carried out on finches from the Galapagos Islands. Finches, like other wild birds, might be proposed as sentinels of AMR in the archipelago. In this study, we observed a high AMR percentage towards tetracycline in *E. coli* and *Enterococcus* isolates, especially in the agricultural zone of Santa Cruz Island. It is known that the antimicrobials usually used as therapeutic and prophylactic agents in production animals are absorbed only in low amounts by the gut of animals. Moreover, tetracycline is mostly evacuated as an active compound within excreta. In this way, livestock manure, which is widely used as fertilizer in agriculture, may act as an important reservoir of AMR genes in the soil of the agricultural zone, due to anthropogenic practices exerted on the island. Also, AMR bacteria have been found in high levels in manure from animals with no history of antibiotic use due to the bacteria intrinsically resistant to antimicrobials harbored in their intestinal tracts. In our study, we were able to determine circulating MDR bacteria as well as AMR profiles in *E. coli* and *Enterococcus* spp. isolates. However, further studies should be carried out to clarify the AMR patterns circulating in the Galapagos islands, as well as to better understand the role of anthropogenic variables in the development of AMR.

Acknowledgment

The present study was supported by the Galapagos National Park Direction (DPNG) and the Agency for the Regulation and Control of Biosafety and Quarantine for the Galapagos (ABG). We are grateful to María Belén Cevallos, Carlos Gómez, José Luis Medina, and Sofia de Janon for their contribution to this study. We also thank Clara López for her help in the statistical data analysis.

Funding Information

This study was funded by the Universidad Central del Ecuador, Ecuador. Sampling logistics at Galapagos were provided by the ABG.

Author's Contributions

María Inés Baquero: Design of the study, field and laboratory work, data analysis, and manuscript writing.

Marylin Cruz: Head of logistics in the Galapagos Islands.

Viviana Duque: Sample logistics in Santa Cruz Island.

Alberto Velez: Sample and permission logistics in Santa Cruz Island.

Vanessa Lopez: Contributed to field and laboratory analyses.

Christian Vinueza: Conceptualization, methodology, supervision, and manuscript writing.

Gabriela Giacoboni: Conceptualization, methodology, supervision, contribution to laboratory work, and manuscript writing.

Ethics

This study was approved by the Institutional Committee for the Care and Use of Laboratory Animals of the Universidad Nacional de La Plata, Buenos Aires, Argentina (88-2-18T). All samples were collected under the Contra to Marco MAE-DNB-CM-2028-0094 and the DPNG authorization No PC 85-18.

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