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PHENOTYPIC RESISTANCE PROFILES OF SALMONELLA ENTERICA ISOLATED FROM WILD FELIDS IN COSTA RICA BETWEEN 2021 AND 2022

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Abstract: *Salmonella* spp. are one of the leading causes of illness, and in the last years there is an increasing interest in the role of different wild animals as reservoir of *Salmonella enterica*, especially multidrug resistant strains. To establish preventive and action strategies, it is essential to monitor bacterial resistance profiles and systematically collect information. This study aims to report *Salmonella enterica* and their resistance profile isolated from feces of wild felids that receive veterinary cares by the Hospital de Especies Menores y Silvestres, Costa Rica in 2021 and 2022. Overall, 100% (7/7) of *Salmonella* spp. isolates exhibited resistance against cefazoline, followed by 71% (5/7) to ciprofloxacin, and 43% (3/7) to nitrofurantoin. A single isolate was found to be multidrug resistant against Ampicillin/Sulbactam-Cefazolin-Ceftriaxone-Gentamicin-Ciprofloxacin-Nitrofurantoin. These resistant profiles highlight that *Salmonella enterica* isolation can represent a threat to public health and wildlife conservation, especially for those organisms expressing resistant phenotypes to drugs commonly used in clinical settings. Fluoroquinolone resistant *Salmonella* spp. have been called by the World Health Organization a high priority for research. As these organisms are expanding beyond livestock and hospital associated environments, to understand the epidemiology and impact of fluoroquinolone-resistant *Salmonella* spp. we require a One Health approach.

INTRODUCTION

Salmonella spp. are one of the leading causes of foodborne illness, and in the last years, there is an increasing interest in the role of wild animals as reservoir for *Salmonella enterica*.[10] Especially for multidrug-resistant strains, which are not expected to be recovered from wildlife.[22] There is a gap of information available describing the role of wild felids as reservoirs of *Salmonella*. Many animals may be subclinical carries of this organism, which represents a threat to public health and wildlife conservation. The risk of wild felids acquiring and shedding *Salmonella* and resistant bacteria is high because of their carnivorous diet as top predators or being fed raw meat under human care.[18,22] Wildlife became colonized with antibiotic-resistant bacteria through feed, water, environments, human contact, or contact with other colonized animals amplifying the antimicrobial resistance issue.[3] Furthermore, if an animal becomes ill with a resistant bacteria, these resistant phenotypes may limit the antibiotic options to treat the infection.[2] The global increasing frequency of *Salmonella* resistant strains to antibiotics makes the challenge arduous.[8] The problem of antibiotic resistance does not respect social, economic, biological, or geographical barriers. To establish preventive and action strategies, it is important to monitor bacterial resistance profiles and systematically collect information.[6,7] Here, we aim to report the prevalence and resistant profiles of *Salmonella enterica* isolated from the feces of wild felids attended by the Hospital de Especies Menores y Silvestres (HEMS) Universidad Nacional de Costa Rica (UNA) from 2021–2022.

MATERIALS AND METHODS

From 2021 and 2022, a screening for *Salmonella enterica* and their phenotypic antibiotic resistant profiles was carried out in all the 22 wild felids receiving medical attention in the HEMS-UNA, Heredia, Costa Rica. The HEMS-UNA is an academic veterinary teaching hospital and reference center dedicated to providing veterinary care to wildlife species *in situ* and *ex situ* in Costa Rica, giving services to the Costa Rican government and private wildlife centers. This study was done according to the regulations of the National Committee of Biodiversity from the Ministry of Environment and Energy (CONAGEBIO-MINAE) and approved in permit R-CM-UNA-005-2021-OT-CONAGEBIO. A total of 22 wild felids received medical attention by HEMS from 2021-2022. All of them were screened for *Salmonella enterica*. Feces were collected from them upon admission, regardless of whether healthy

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Table 1. Frequency of *Salmonella enterica* recovered from wild felids attended by HEMS-UNA between 2021 and 2022.

Species	Free-range[1]	Under Human Care[2]	Total (%)	Resistant - Profiles
Puma concolor costarricensis	1/1	1/5	2/6 (33,3%)	CZ-CIP[1] CZ-CIP-NIT[2]
Panthera onca	0/1	1/4	1/5 (20%)	SAM-CZ-CRO-GM-CIP-NIT[2]
Leopardus pardalis	0/1	2/6	2/7 (28,6%)	CZ-CIP[2] CZ-CIP-NIT[2]
Herpailurus yagouaroundi	1/1	—	1/1 (100%)	CZ[1]
Leopardus wiedii	—	1/3	1/3 (33,3%)	CZ[2]
Total (%)	2/4 (50%)	5/18 (27,8%)	7/22 (31,8%)	

or not. Although the animals were sedated for clinical evaluation or surgical procedure, a fresh fecal sample was taken directly from each animal in a sterile container. Immediately, the samples were transferred to the Public Health and Food Safety Laboratory of the School of Veterinary Medicine of the National University to carry out the *Salmonella* culture.

Four grams of feces were cultured in 36 mL of Tetrathionate broth (BD Co., Spark, MD 21152, USA) to which iodine was added in a ratio of 1:20, followed by incubation for 18-24 h in a water bath at 37°C. Then, 0.1 mL of the inoculum was placed in 10 mL of Rappaport-Vassiliadis broth (BD Co., Spark, MD 21152, USA) and incubated in a water bath at 42°C. After 24 h, the inoculum was plated on Xylose-Lysine-Tergitol-4 (XLT-4) agar (Remel, Lenexa, KS 66219, USA) and incubated for 18-24 h at 37°C. *Salmonella* Abaetetuba (ATCC 35640), H2S(+) was used as a positive control, *S*. Cholerasuis (ATCC 10708), H2S(-), and *Escherichia coli* (ATCC 25922) were used as negative controls. A single colony per plate, black or black in the center with a yellow periphery, compatible with *Salmonella*, was transferred to MacConkey (Mck) agar (BD Co., MD 21152, USA). Lactose-negative colonies with morphologic characteristics compatible with *Salmonella* spp. were placed in 5 mL of Triple-Iron-Sugar agar (TSI) (BD™, Le Pont de Claix, France) and Lysine-Sugar Iron Agar (LIA) (Sigma-Aldrich, MO 63178, USA), at 37°C for 24 h. Samples with a positive reaction in TSI and LIA agar, and visual glass agglutination test using a polyvalent *Salmonella* antiserum (Denka Seiken Ltd., Tokyo 103-8338, Japan) were considered as *Salmonella* spp. To confirm these isolates, VITEK®2 GN cards (BioMérieux, Capronne 69290, France) were used. The antibiotic susceptibility profiles were obtained using Minimum Inhibitory Concentrations (MIC) with VITEK®2 AST-N401 for MIC concentration (BioMérieux, Capronne 69290, France). The antibiotics tested included: Ampicillin/Sulbactam (SAM), Cefazoline (CZ), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefepime (FEP), Ertapenem (ERT), Meropenem (MEM), Amikacin (AN), Gentamicin (GM), Ciprofloxacin (CIP), Nitrofurantoin (FM), and Trimethoprim/Sulfamethoxazole (SXT). A bacterial concentration equivalent to 0.5 according to the McFarland standard in 3 mL of 0.45% sterile saline (Prelab, San José, Costa Rica) for additional testing 145 μL of this solution to 3 mL of sterile 0.45% saline (BioMérieux, Capronne 69290, France). The first solution was used for the GN cards and confirmation of *Salmonella enterica*. The second solution was used to obtain the susceptibility profile with AST-N279 cards. Isolates were classified by their MIC as susceptible and non-susceptible or resistant. Intermediate breakpoints were interpreted as non-susceptible. MICs were analyzed according to the Clinical Laboratory Standards Institute breakpoints.[4] Because of small sample size, Fisher exact test was used to determine if there was an association with *Salmonella* spp. status, sex, and location of felid (under human care vs wild). A *p* value of 0.05 was considered significant.

RESULTS

In this sample size a 32% (7/22, 95% Confidence interval: 16.4-52.7%) of *Salmonella enterica* was recovered from all wild felids presented to the HEMS from 2021 to 2022. The microorganism was isolated from five of the six wild felid species found in Costa Rica. A total of seven ocelots (*Leopardus pardalis)* (2/7. 29%, 95% Confidence interval: 8.2-64.1%), six pumas (*Puma concolor costarricensis)* (2/6. 33%, 95% Confidence interval: 9.7-7.0%), five jaguars (*Panthera onca)* (1/5. 20%, 95% Confidence interval: 3.6-62.4%), three margays (*Leopardus wiedii*) (1/3. 33%, 95% Confidence interval:6.1-79.2%) and one jaguarundi (*Herpailurus yagouaroundi)* (1/1. 100.0%, 95% Confidence interval: 20.7-100.0%) were screened for *Salmonella enterica* (Table 1). No oncilla (*Leopardus tigrinus)* were screened during this period. From free range felids, 50% (2/4) of the samples

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were positive, whereas 27.8% (5/18) from the animals under human care. In terms of sex, 33% of males (4/12) and 33% of females (3/10) were positive.

Overall, 100% (7/7) of *Salmonella* spp. isolates exhibited resistance against cefazoline, followed by 71% (5/7) to ciprofloxacin, and 43% (3/7) to nitrofurantoin. A single isolate was found to be multidrug resistant against Ampicillin/Sulbactam-Cefazolin-Ceftriaxone-Gentamicin-Ciprofloxacin-Nitrofurantoin (Table 2).

DISCUSSION

Salmonella enterica isolation can represent a threat to public health and wildlife conservation, especially when it is isolated from asymptomatic carriers and expressing resistant phenotypes that can represent an issue to treatment options for both human and animal patients.[17] *Salmonella* has been already reported to cause disease and mortality in wild felids.[14,18] The prevalence of *Salmonella enterica* from wild felids can be >90%, including a high prevalence of antibiotic resistant profiles.[5] In this current report, prevalence was 32%, but based on the limited sample size and 95% CI (16.4-52.7%) could have been >50% in this population of animals in Costa Rica. There is no statistically significant difference association between the variables *Salmonella* spp. status and location of felid (under human care vs wild) ($p = 0.79$), and sex ($p = 0.88$). This prevalence still represents a significant risk to caretakers, wildlife and livestock/domestic species these felids may encounter. It has been suggested that these felids usually got colonized and infected by feed (raw meat), prey, water, and/or contaminated environments.[5,14,18] The differences between the prevalence found in this report and earlier research involving screening of wild felines can be attributed to factors like as dietary composition and feed handling, environmental conditions, sample collection, and the microorganisms' capacity to persist as a normal component of the gastrointestinal flora.[5] In Costa Rica, resistant genes identified from wild felid feces have been associated with the proximity of livestock production and anthropogenic activities.[1] It is complicated to determine when and where the animals got colonized by *Salmonella* or exposed to antibiotics wastes. But these screened animals were associated with proximity to livestock and areas with high human density. Areas in where dense human or livestock populations are established are associated with multiple sources and amplifiers for antimicrobial resistance, including wildlife.[2] One of the main threats wild

Table 2. Resistant profiles based on minimum inhibitory concentration (MIC) values of 7 isolates recovered from feces in wild felids attended by HEMS-UNA between 2021 and 2022.

Class	Antimicrobial (g/mL)	0.1	0.3	0.06	0.12	0.25	0.5	1	2	4	8	16	20	32	64	76	128	256	512	Total
Beta Lactam	Ampicillin sulbactam								6			1								1
	Cefazolin									6					1					7*
	Ceftazidime					2		4		1										0
	Ceftriaxone					6								1						1
	Cefepime				2			4												0
	Imipenem				7															0
	Meropenem					7														0
Aminoglycosides	Amikacin							1	5	1										0
	Gentamicin							6			1									1
Quinolone	Ciprofloxacin			2			4			1										5
Nitrofuran	Nitrofurantoin											1		3	2				1	3
Folate Antagonist	Trimethoprim sulfamethoxazole												7							0

The number in the table represents the number of isolates according to their MIC for each antibiotic. Vertical black lines represent the cut-off point for the characterization of susceptibility, susceptible to the left and non-susceptible to the right. Green color represents susceptible isolates, and gray represents non-susceptibles[9]. * U.S. Food and Drug Administration (2022). Rationale for FDA's Position on the Use of Cefazolin Breakpoints as a Surrogate for Determining Breakpoints for Oral Cephalosporins for the Treatment of Uncomplicated Urinary Tract Infections.

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felids in Costa Rica face are the livestock-wild felids conflicts. Wild felids are usually classified as pests by some ranchers because they eat their livestock.[15] This predation factor, spacial distribution around farms and being fed by livestock when they lived under human care can represent a risk factor to acquire resistant bacteria and genes. The finding of resistant phenotypes in this report, especially ciprofloxacin and a multidrug resistant isolate is alarming. Isolation of CIP and FM resistant profiles matches with *Salmonella* isolates reported from non-human primates,[17] human clinical cases,[20] and food in Costa Rica.[9,13] The multidrug resistant phenotype is probably related to the raw meat diet under human care. Diet that is based on raw meat from calves, chicken and pork where these resistance profiles are reported in Costa Rica.[9,12,13] Fluoroquinolone-resistant *Salmonella* strains are considered by the World Health Organization a high priority for research.[19] Enrofloxacin is a third-generation fluoroquinolone that metabolizes in CIP in some animal species.[21] For human medicine, ciprofloxacin is available only with a doctor's prescription. However, in most veterinary pharmacies in Costa Rica, people can buy enrofloxacin without restrictions. Enrofloxacin is less expensive and easier to access than other antibiotics in veterinary pharmacies, leading to its broad empirical use in the animal population. This lack of antibiotics regulation, easy access to enrofloxacin and broad-spectrum activity makes it widely used and misused in animals (Livestock and domestic animals) in Costa Rica. This fluoroquinolone excretion and wastes leaking into the environment are probably generating selective pressure in bacteria like *Salmonella* contributing to fluroquinolone resistant phenotypes appearance. This could lead to the emergence of resistance mechanisms. Chromosomal mutations in genes encoding the subunits of the drugs' target enzyme (DNA gyrase and topoisomerase IV) are one of the main mechanisms[11]. The high levels of resistance to CIP threaten its use as a treatment for invasive salmonellosis in humans, which has led to the use of drugs such as CRO.[16] Isolating *Salmonella* resistant to both CRO and CIP drugs generates a health alert about the expansion of these multiresistant isolates in wildlife.[3] On the other hand, CZ is evaluated for research purposes and antimicrobial resistant surveillance, it is not an antibiotic reported routinely for *Salmonella* spp. First- and second-generation cephalosporins should not be reported as susceptible for clinical purposes.[4] The high prevalence of resistance to ciprofloxacin and isolation of a multi drug resistant strain identified in these samples is a concern for global health. Wild felids, especially free-range felids, can serve as indicators of antimicrobial resistance. Further research should address epidemiological surveillance of *Salmonella,* nitrofurantoin and fluoroquinolones resistant strains, and fluoroquinolones wastes in the environment to mitigate this complex problem in Costa Rica. Understanding the epidemiology of fluoroquinolone-resistant *Salmonella* requires a One Health approach, as they are spreading in Costa Rica outside livestock, community, and hospital-associated environments.[17] This study has certain limitations and bias, such as the sample size, selection bias of individual from only one healthcare center. Temporality and recall bias were also identified when trying to get their clinical history and possible exposures since most of the animals their history was sacred. Multidisciplinary ecology and epidemiological studies must be conducted for better comprehension of the risk and determinants of antimicrobial resistance and its spread to develop evidence-based antibiotic usage policies. The education of the population on the use of antibiotics must be reinforced. To complete these strains characterization, further analysis of these isolates like whole genome sequencing must be done to identify antimicrobial resistant genes, serotypes, and virulence factors.

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