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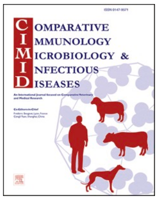

Antimicrobial resistance patterns of bacteria isolated from chicks of Canarian Egyptian vultures (*Neophron percnopterus majorensis*): A "one health" problem?

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ABSTRACT

Antimicrobial resistance in Gram-negative bacteria isolated from cloacal samples of chicks of Canarian Egyptian vultures was investigated. Prevalence of *Salmonella* was also studied. Forty-seven isolates, obtained from 23 animals, were analysed. *Escherichia coli* (n = 29), *Proteus mirabilis* (n = 17) and *Salmonella* spp. (n = 1) were identified using API 20E system. Antimicrobial susceptibility to 13 antibiotics included in nine different categories was determined using disk-diffusion technique. The higher percentages of susceptible *E. coli* isolates were found for aminoglycosides and cefoxitin, and the lower ones were found for ampicillin, enrofloxacin and tetracycline. *Proteus mirabilis* isolates were susceptible to most of antimicrobials tested. Multidrug resistance patterns were found in 13 *E. coli* and four *Proteus mirabilis*. *Salmonella* spp. was detected in one chick (4.37%), and the isolate was also resistant to ampicillin and tetracycline. Thirteen *E. coli* isolates and four *Proteus* isolates showed a multidrug-resistance pattern, being resistant at least to one antibiotic in three or more different antimicrobial categories. This high level of antibiotic resistance in chickens of an endangered bird may be a limitation for possible treatments of infections in this species, as well as representing a source of resistant bacteria for animal care staff and for other animals in wildlife recovery centres. A "One Health" approach to this problem is necessary to reduce the levels of antimicrobial resistance in wild birds.

1. Introduction

Antimicrobial resistance is a silent pandemic that represents a huge global health problem. Through different pathways, antimicrobial resistant (AMR) bacteria or genes conferring antimicrobial resistance, are transferred from human and domestic animals to wild animals and environment and back [1,2]. In wild animals, antimicrobial resistance is being increasingly reported worldwide [3–5]. Some wild animals, specially synanthropic birds, are especially prone to acquire antimicrobial resistant bacteria, due to their contact with wastewater and landfills [6,7]. All these elements represent important factors in the "One Health" network [8,9].

Several definitions of Multidrug-resistant (MDR) bacteria have been proposed. At the initiative of the European Centre for Disease Control and Prevention (ECDC) and the US Centres for Disease Control and Prevention (CDC), an expert group was established to standardise the terms used to describe resistance profiles [10]. The proposal was made mainly for antimicrobials used in humans' infections, defining categories of antibiotics used for treatment of infections caused by different groups of bacteria. An isolate is considered MDR when shows acquired non-susceptibility to at least one agent in three or more antimicrobial categories. Non-susceptibility refers to either a resistant, intermediate or non-susceptible result obtained from in vitro antimicrobial susceptibility testing. Some species have intrinsic resistance to certain antibiotic or

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categories of antibiotics. If this happens, that antibiotic or category should not be considered when calculating the number of agents or categories to which the isolate is non-susceptible. The Canary Islands (Spain), located in the northwest of the African coast and 100 km from the coast of Western Sahara, have a human population of approximately 2100,000 inhabitants. The chicks of the Canarian Egyptian vultures, the scavenger birds that are the subject of this study, live on the islands of Fuerteventura and Lanzarote, although the main nesting area is the mountainous centre of the island of Fuerteventura. These birds fly easily between the two islands for feeding because of they are very close (10 km). In addition to the supplementary feeding station located near the nesting areas of the vultures on the island of Fuerteventura (Fig. 1), there are two landfills on each of these islands. Specifically, the Fuerteventura landfill is located about 20 km away from the main nesting area (Fuerteventura) and the Lanzarote landfill is located about 130 km away.

The Canarian Egyptian vulture (*Neophron percnopterus majorensis*) is an endemic raptor of the Canary Islands. It is included under the category of "In Danger of Extinction" in the Spanish Catalogue of Endangered Species [11] and under the category of "Endangered" in the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species. These vultures are not migratory, and movement is limited to the islands they inhabit, so their role in the worldwide dissemination of MDR bacteria is less relevant than the one of migratory birds [12]. It is, mainly, a necrophagous bird. Vultures, as other raptors, can be exposed to antibiotics via livestock carcasses and small animals' ingestion, in addition to environmental exposure.

The aim of our study was to assess the presence of AMR bacteria in chicks of Canarian Egyptian vultures when they are still in nest and, therefore, not directly exposed to environmental sources of antimicrobials.

2. Material and methods

Within a long-term monitoring program of Canarian Egyptian vultures (*Neophron percnopterus majorensis*) [13], chicks of vultures were captured in nests during the fledgling stage. There was only one chick per nest and all chicks born in 2019 were included. All of them were apparently healthy and they had not received any antimicrobial treatment. The capture, handling, and sampling methods of wild vultures were carried out under Project Licence approved by Dirección de Biodiversidad del Gobierno de Canarias (Canary Islands Government); competent authority official reference numbers 2014/256, 2015/1652 and 2016/1707.

A total of 23 cloacal samples were obtained, each one from a different animal, using a sterile cotton swab. Cloacal swabs were placed in Amies transport medium (Copan Italia, Brescia, Italy) and conserved at 4ºC until they reached to the Microbiology Laboratory (within 24 h).

Samples were cultured on MacConkey agar (BD Difco, Detroit, MI, USA), McConkey + CTX ($2\ \mu g\ ml^{-1}$) agar and *Salmonella-Shigella* agar (BD Difco) and incubated overnight at 37 °C.

Fig. 1. Canary vultures feeding at the supplementary feeding station located near the vulture nesting areas on the island of Fuerteventura.

Selenite Broth (BD Difco) was also inoculated and incubated overnight at 37° C and later streaked onto *Salmonella-Shigella* agar.

Bacteria were identified using API 20E test (bioMérieux, Marcy L'Etoile, France), following the manufacturer's instructions.

Antimicrobial susceptibility tests were performed using the disk diffusion method on Müller–Hinton agar plate (BD Difco) in accordance with the Clinical and Laboratory Standards Institute (CLSI 2017) guidelines [14]. The inhibition zones were measured, and the isolates were classified as susceptible, intermediate, or resistant, according to the guidelines established by the CLSI. Antibiotic categories and disks (BD BBL, Sparks, MD, USA) used are summarized in Table 1.

3. Results

Forty-seven Gram-negative isolates were recovered: from 23 animals: 61.7% *E. coli* (29/47), 36.2% *Proteus mirabilis* (17/47) and 2.1% *Salmonella* spp. (1/47). Using API 20E system, seven different biochemical profiles were found for *E. coli*, being the most frequent 5144572, with 21/29 (72.4%) isolates presenting that one. For *Proteus mirabilis*, two different profiles were detected, and 15/17 (88.2%) isolates showed one of them (0736000).

Twenty-three (79.3%) *E. coli* isolates grew in McConkey Agar and six grew in MC+CTX. Nineteen different antimicrobial resistance patterns were detected for *E. coli,* being 6/29 (20.69%) isolates susceptible to all antimicrobials tested. Thirteen isolates (44.8%) meet the definition of MDR. One isolate was resistant to all eight antimicrobial categories tested. Resistance to six categories of antimicrobials was detected in three isolates; to five categories in four isolates; to four categories in four isolates and to three, in oner isolate. Molecular characterization of antimicrobial resistance of the *E. coli* strains isolated from MC+CTX plates have been described in a previous paper [15].

For *Proteus mirabilis*, eight different resistance patterns were found. Four isolates (23.5%) were MDR. One isolate was resistant to five different antimicrobial categories, two isolates were resistant to four categories and one, to three categories.

Antimicrobial resistance patterns and number of antimicrobial categories included in them are shown in Table 2.

Percentages of susceptibility to different antibiotics are presented in Table 3. More than 90% of *E. coli* isolates were susceptible to aminoglycosides or cefoxitin. The lower percentages of susceptible isolates were found for AM and TE.

Proteus mirabilis isolates were, in general, susceptible to most of the antibiotics tested. Lower percentages of susceptibility (70.6%) were found to AM, ENO and SXT.

Table 1
Antimicrobial categories and antimicrobial agents tested.

Antimicrobial categories	Antimicrobial agents	Abbreviation and charge of disks
Aminoglycosides	Amikacin	AN (30 μg)
	Gentamicin	GM (30 μg)
	Tobramycin	NN (10 μg)
Extended spectrum cephalosporins: 3rd and 4th generation cephalosporins	Cefotaxime	CTX (30 μg)
	Ceftazidime	CAZ (30 μg)
Cephamycins	1. Cefoxitin	2. FOX (30 μg)
Fluoroquinolones	Ciprofloxacin	CIP (5 μg)
	Enrofloxacin	ENO (5 μg)
Folate pathway inhibitors	3. Trimethoprim/ Sulfamethoxazole	4. SXT (1.25 μg + 23.75 μg)
Penicillins	5. Ampicillin	6. AM (10 μg)
Penicillins + β-lactamase inhibitors	Amoxicillin / Clavulanic Acid	7. AMC (20 μg + 10 μg),
Phenicols	8. Chloramphenicol	9. C (30 μg)
Tetracyclines	10. Tetracycline	11. TE (30 μg)

Table 2
Antimicrobial resistance patterns.

Patterns of antimicrobial resistance	Number of antimicrobial categories included in the resistance pattern	Number of isolates
E. coli		
AM	12. 1	13. 1 (3.4%)
AMC, ENO, TE, GM, CTX, AM	14. 6	15. 1 (3.4%)
AMC, TE, AM	16. 3	17. 1 (3.4%)
AN, AM	18. 2	19. 1 (3.4%)
CAZ, AMC, FOX, CTX, AM	20. 4	21. 1 (3.4%)
CAZ, CTX, AM	22. 2	23. 1 (3.4%)
ENO, CIP	24. 1	25. 1 (3.4%)
ENO, TE, CIP	26. 2	27. 1 (3.4%)
SXT, ENO, TE, CIP, AM	28. 4	29. 3 (10.3%)
SXT, AM	30. 2	31. 1 (3.4%)
SXT, AMC, ENO, TE, AM	32. 5	33. 1 (3.4%)
SXT, AMC, ENO, TE, C, CIP, AM	34. 6	35. 1 (3.4%)
SXT, AMC, ENO, TE, CIP, AM	36. 5	37. 2 (6.9%)
SXT, AMC, TE, AM	38. 4	39. 1 (3.4%)
SXT, CAZ, AMC, ENO, TE, CTX, AM	40. 5	41. 1 (3.4%)
SXT, CAZ, AMC, ENO, TE, FOX, C, CIP, CTX, AM	42. 8	43. 1 (3.4%)
SXT, CAZ, ENO, TE, C, CIP, CTX, AM	44. 6	45. 1 (3.4%)
TE	46. 1	47. 3 (10.3%)
Susceptible to all antimicrobials tested	–	49. 6 (20.7)
Proteus mirabilis[*]		
SXT, ENO, TE, C, AM	50. 4	51. 1 (5.9%)
SXT, ENO, TE, C, CIP, AM	52. 4	53. 1 (5.9%)
SXT, ENO, TE, C, CIP, CTX, AM	54. 5	55. 1 (5.9%)
ENO, TE, AM	56. 2	57. 1 (5.9%)
ENO, TE, CIP	58. 1	59. 1 (5.9%)
SXT, TE, C, AM	60. 3	61. 1 (5.9%)
SXT, TE	62. 1	63. 1 (5.9%)
TE	–	65. 10 (58.8%)
Salmonella spp.		
TE, AM	66. 2	67. 1 (100%)

[*] *Proteus* is intrinsically resistant to TE, so this resistance is not considered when counting antimicrobial categories included in the resistance pattern.

Salmonella spp was detected in one chick (4.37%) and was resistant to AM and TE.

4. Discussion

Antimicrobial resistance is a "One Health" issue [9]. Resistance in environmental bacteria is increased by the transference of AMR bacteria or genes from human and livestock waste bacteria, and for the high quantities of antimicrobial residues that enter to environment from different sources, creating a high selective pressure. Wildlife is also exposed to these sources of antimicrobial resistance, through environmental exposure or through direct ingestion of other animals [16]. To close the circle, human beings could be infected by AMR bacteria from wild or domestic animals [17,18].

Wild birds can act as reservoirs and spreaders of AMR bacteria [1]. Some studies have demonstrated that AMR bacteria carried by wildlife usually correlate with anthropogenic sources of pollution [6,7,19,20].

E. coli was recovered in samples from all animals tested, a higher prevalence than the one described before [5,21].

The prevalence of *Salmonella* in our study was 4.34%, similar to the one found by other authors [5,22,23] for adult animals and chicks. It has been described no differences in prevalence of *Salmonella* between birds of different ages [5].

From chicks, eighteen different *E. coli* susceptibility patterns and eight different *Proteus mirabilis* ones were detected. MDR was detected in 44.8% of *E. coli* isolates and 22.5% of *Proteus mirabilis* isolates. In 2017, Borges et al. [24] described 47.4% MDR *E. coli* isolates from wild birds at a veterinary hospital in Brazil, none of them suffering from infectious diseases.

In 2021, Gambino et al. [25] found an *Escherichia* spp. prevalence rate of 23.3% in birds in Sicily (Italy), being only two *E. coli* isolates from raptors classified as MDR (4.4%). Both, the prevalence of *E. coli* and the rate of MDR strains described were much lower than those found in our study. In other study in Catalonia, a prevalence of 7.3% of cephalosporin-resistant (CR) Enterobacterales in raptors was found [26], lower than ours (about 16%). Among all the samples tested at a wildlife rehabilitation centre, they found 45% of CR *E. coli* and 5% of CR *Proteus mirabilis*, but when considering only raptors, 12.96% CR *E. coli* and 1.85% CR *Proteus mirabilis* were obtained. We found 20.6% and none respectively. Chandler et al. [2] found prevalence of 4% of *E. coli* growing in CTX-MC among faecal samples of European starlings, lower than the one found in our study (26%). The differences described are surprising since our study includes only strains obtained from nestlings and, moreover, apparently healthy. It has been described that AMR is more common among in-care birds than in wild birds [27] and in adults than in chicks [5]. According to other results published in the literature, three main hypotheses could explain our results. Firstly, motivated by the presence of multi-resistant bacteria in the food brought by the vulture adults to the chicks (dead animals, leftover food from landfills) (Fig. 2) that has been described previously [28,29]; secondly, because there are traces of antibiotics in the animals or in the rubbish that serves as food for the chicks, and may produce selective pressure on the intestinal bacteria of the chicks. The presence of antibiotic in vultures has been described by several authors in the literature [28,30–33].

The third hypothesis about the origin of these multi-resistant bacteria would be the contact of the chicks in the nest with faeces from the adult vultures. This last hypothesis has been showed by our research team. Adults vultures have *E. coli* and other multi-resistant bacteria in their intestine [5].

A high percentage of *E. coli* isolates (44.9%) were resistant to Enrofloxacin, similar to the one described for common buzzards [34]. In 2020, Chandler et al. [2] found a 10% of quinolone resistant *E. coli* in starlings and Mukerji et al. [35] described 23.8% of fluoroquinolone resistant *E. coli* in Australian gulls. In a subsequent study [36], they also analysed the resistance to several antibiotics among samples from different birds, founding ciprofloxacin resistance percentages of 48% in gulls and over 60% on samples from feral pigeons and little penguins. High percentages of isolates resistant to AM, TE and SXT were also found. These results are similar to those found in our study. In a study done in Brazil [24], resistance rates were similar to ours for some antimicrobials (TE, SXT, C, CIP) but very different for aminoglycosides and beta-lactams. These differences could be attributed to the inclusion of a wide variety of birds in the Brazilian study (mostly non-predatory birds such as swans, parrots, parakeets, pigeons, etc.) or due to the presence of disease at the time of sampling and, to a lesser extent, different antibiotic use protocols in the region. In 2021, Nowaczek et al. [37] found percentages of resistance to TE, SXT and CIP like our results, but very different ones for GM, AM or C. They found a very high percentage of strains resistant to GM (75%) which is different than ours (3.5%) and that described by other authors [38].

Proteus mirabilis resistant isolates usually showed resistance to two antimicrobial groups and commonly used to treat infections caused by this species in animals (sulphonamides and quinolones) but not to other (aminoglycosides) also used for veterinary treatments of these infections. Schultz et al. [39] found a high proportion of *Proteus mirabilis* isolates resistant to chloramphenicol (77.8%), trimethoprim/ sulphonamides (72.2%) and enrofloxacin (55.6%), much higher than those found in our study.

In a recent study, [39] a 4% prevalence rate of ESBL/AmpC in *Proteus mirabilis* of animal origin (mainly dogs) was described. We found only

Table 3
Percentage of resistant, intermediate and susceptible isolates (*E. coli* n = 29) isolates; *P. mirabilis* n = 17 isolates; *Salmonella* n = 1 isolate.).

Antibiotic	Species	Resistant (%)	Intermediate (%)	Susceptible (%)
Ampicillin	68. *E. coli*	69. 18 (62.1)	70. 1 (3.4)	71. 10 (34.5)
	72. *P. mirabilis*	73. 3 (17.6)	74. 2 (11.8)	75. 12 (70.6)
	76. *Salmonella*	77. 1 (100)	–	–
Amoxicillin/ Clavulanic Acid	80. *E. coli*	81. 2 (6.9)	82. 8 (27.6)	83. 19 (65.5)
	84. *P. mirabilis*	–	–	87. 17 (100)
	88. *Salmonella*	–	–	91. 1 (100)
Cefoxitin	92. *E. coli*	93. 2 (6.9)	–	95. 27 (93.1)
	96. *P. mirabilis*	–	–	99. 17 (100)
	100. *Salmonella*	–	–	103. 1 (100)
Cefotaxime	104. *E. coli*	105. 6 (20.7)	–	107. 23 (79.3)
	108. *P. mirabilis*	–	110. 1 (5.9)	111. 16 (94.1)
	112. *Salmonella*	–	–	115. 1 (100)
Ceftazidime	116. *E. coli*	117. 3 (10.3)	118. 2 (6.9)	119. 24 (82.8)
	120. *P. mirabilis*	–	–	123. 17 (100)
	124. *Salmonella*	–	–	127. 1 (100)
Gentamicin	128. *E. coli*	129. 1 (3.5)	–	131. 28 (96.5)
	132. *P. mirabilis*	–	–	135. 17 (100)
	136. *Salmonella*	–	–	139. 1 (100)
Tobramycin	140. *E. coli*	–	–	143. 29 (100)
	144. *P. mirabilis*	–	–	147. 17 (100)
	148. *Salmonella*	–	–	151. 1 (100)
Amikacin	152. *E. coli*	153. 1 (3.5)	–	155. 28 (96.5)
	156. *P. mirabilis*	–	–	159. 17 (100)
	160. *Salmonella*	–		162. 1 (100)
Enrofloxacin	163. *E. coli*	164. 10 (34.6)	165. 3 (10.3)	166. 16 (55.1)
	167. *P. mirabilis*	168. 5 (29.4)	–	170. 12 (70.6)
	171. *Salmonella*	–	–	174. 1 (100)
Ciprofloxacin	175. *E. coli*	176. 9 (31.1)	177. 1 (3.4)	178. 19 (65.5)
	179. *P. mirabilis*	180. 3 (17.6)	–	182. 14 (82.4)
	183. *Salmonella*	–	–	186. 1 (100)
Tetracycline	187. *E. coli*	188. 16 (55.2)	–	190. 13 (44.8)
	P. mirabilis[a]	191. 17 (100)	–	–
	194. *Salmonella*	195. 1 (100)	–	–
Chloramphenicol	198. *E. coli*	199. 3 (10.3)	–	201. 26 (89.7)
	202. *P. mirabilis*	203. 4 (23.5)	–	205. 13 (76.5)
	206. *Salmonella*	–	–	209. 1 (100)
Trimethoprim/ Sulfamethoxazole	210. *E. coli*	211. 12 (41.4)	–	213. 17 (58.6)
	214. *P. mirabilis*	215. 5 (29.4)	–	217. 12 (70.6)
	218. *Salmonella*	–	–	221. 1 (100)

[a] *P. mirabilis* is intrinsically resistant to tetracycline.

Fig. 2. Canary vultures feeding on pig heads coming from the Fuerteventura Island slaughterhouse and brought to the supplementary feeding station.

one strain with intermediate resistance to CTX, therefore no presence of ESBL/ AmpC *Proteus mirabilis* isolates was detected.

The only one *Salmonella* isolate was resistant to AM and TE, a common pattern of antimicrobial resistance in this species [40]. No MDR *Salmonella* isolates were found in another study in wild raptors in Spain [22], but MDR *Salmonella* Infantis human-associated clones have been described in wild owls in South America [3]. Among raptors admitted to a wildlife rehabilitation centre (n = 121) in Catalonia, 10% of prevalence of *Salmonella* was described being 33.3% of them MDR [21].

As concluded by Swift et al. [16] in their study on the anthropogenic environmental drivers of antibiotic resistance in wildlife, the patterns and prevalence of antibiotic resistance are not easily and exclusively attributable to anthropogenic factors. Therefore, further studies are needed to gain a deeper understanding of the mechanisms underlying the origin of antibiotic resistance in wildlife.

5. Conclusion

It could be assumed that wild animals have very low exposure to antimicrobials and therefore we should find low levels of antimicrobial resistance. In the case of nestlings, exposure is even lower and yet we have found relatively high levels of resistant bacteria.

The contact of wild birds with environment (e.g., water, waste, landfills, small rodents, etc.) contaminated with antibiotic residues could be the origin of this resistance.

Our data shown that nestlings can be a reservoir of MDR Enterobacterales and zoonotic bacteria, representing a risk for animal care staff and for other animals in wildlife recovery centres. In addition to this, the presence of MDR bacteria could also make it difficult to treat infections in this endangered species.

Institutional review board statement

The capture and handling methods for wild vultures, were carried out under the Project License approved by The Biodiversity Directorate of the Government of the Canary Islands.

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Conflict of interest

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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