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Brief Report

Molecular diversity of Extended-spectrum β-lactamase-producing *Escherichia coli* from vultures in Canary Islands

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Abstract

Antimicrobial resistance among isolates from wild animals is increasingly reported. Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*, and particularly *Escherichia coli*, have spread worldwide as one of the most common multidrug-resistant organisms. The aim of this study was to determine the carriage rate of ESBL-producing *E. coli* isolates and their genetic characteristics in wild vultures from the Canary Islands. Faecal samples were collected from 22 apparently healthy free-ranging (wild) vulture chicks from Lanzarote and Fuerteventura (Canary Islands) during July 2019. They were seeded in MacConkey agar supplemented with cefotaxime (2 μg ml^{-1}). Colonies with typical morphology of *E. coli* were identified by MALDI-TOF-MS. Antimicrobial susceptibility was done by disk diffusion. Phenotypic detection of ESBL was performed by double-disk tests. The presence of *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{KPC}$ and *bla*$_{OXA-48}$ genes, as well as *mcr*-1 (colistin resistance), *tet*A/*tet*B and *int*1 gene, was tested by PCR/sequencing. Phylogenetic groups and multilocus sequence typing (MLST) were determined by PCR/sequencing. ESBL-producing *E. coli* isolates were detected in 5/22 tested animals (22.7%), and all isolates (one/animal) carried *bla*$_{CTX-M}$ genes: *bla*$_{CTX-M-15}$ (*n* = 3) and *bla*$_{CTX-M-55}$ (*n* = 2). ESBL-positive isolates were ascribed to phylogenetic group D (two isolates), B$_1$ (two isolates) and A (one isolate), and five sequence types were detected (ST/phylogenetic-group/ESBL): ST515/B1/CTX-M-15, ST1290/A/CTX-M-15, ST38/D/CTX-M-15, ST457/D/CTX-M-55 and ST6448/B$_1$/CTX-M-55; this suggests a genetic diversity among these isolates. Three CTX-M-15-producing isolates contained the *bla*$_{TEM}$ gene and one the *tet*A gene. To our knowledge, this appears to be the first report of ESBL-producing *E. coli* in vulture chicks from the Canary Islands.

Introduction

Antibiotics are one of the biomedical revolutions of the 20th century. However, antibiotic resistance constitutes a public health problem nowadays. The excessive use of antibiotics is directly related to the great spread of antibiotic resistance (Pinto *et al*., 2010; Carvalho *et al*., 2019; Ma *et al*., 2017). The abuse and misuse of these drugs can also compromise the prevention and treatment of an increasing range of bacterial infections (WHO, 2017). Therefore, antimicrobial resistance is a 'One Health'

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issue (Robinson *et al*., 2016). 'One Health' is an emerging concept that establish that human, animal and environmental health are interconnected, and a global strategy is needed to face this challenge (Lee *et al*., 2018).

Regarding this situation, different studies have been done in order to understand the mechanisms of bacterial drug resistance in pets and humans (Puño-Sarmiento *et al*., 2013; Leite-Martins *et al*., 2014; Marinho *et al*., 2016; Chung *et al*., 2017; Derakhshandeh *et al*., 2018; Sato *et al*., 2018). Wild birds are also potential reservoirs of resistant bacteria and have the potential to transmit antibiotic resistance, including clinically important resistance genes. During the last two decades, different studies have been published in Europe regarding antimicrobial resistance among wild animals (Costa *et al*., 2008; Poeta *et al*., 2008; Radhouani *et al*., 2012; Gonçalves *et al*., 2014; Dias *et al*., 2015; Alcala *et al*., 2016; Wang *et al*., 2017). Wildlife are not directly exposed to clinically useful antimicrobial agents, but they can acquire resistant bacteria mainly through water polluted from faeces of human and farm activity (Mora *et al*., 2014).

Extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*, and particularly *Escherichia coli*, have spread worldwide as one of the most common multidrug-resistant organisms. Two of the most clinically relevant antimicrobial resistance mechanisms are the production of ESBLs and plasmid-mediated AmpC-type β-lactamases (pAmpC). *Escherichia coli* is a commensal bacterium, which inhabits in the gastrointestinal tract of warm-blooded animals, and can also be a reservoir for antibiotic resistance (Iredell *et al*., 2016; Ulstad *et al*., 2016; Marques *et al*., 2018). The genes encoding these enzymes are frequently plasmid-located and can be horizontally transferred to other bacteria (Alcala *et al*., 2016; Alonso *et al*., 2017). The spread of *E. coli* strains producing CTX-M-type β-lactamases is mostly responsible for the increased incidence of ESBL, especially CTX-M-15 variant, both in animals and humans (Belas *et al*., 2014; Ewers *et al*., 2014; Kojima *et al*., 2014; Rocha-Gracia *et al*., 2015; Zorgani *et al*., 2017).

The Iberian Peninsula holds >90% of the European population of wild vultures (Lopez-Cerero *et al*., 2017). The Egyptian vultures (*Neophron percnopterus*) are the smallest and least dominant of the European vultures (Moreno-Opo *et al*., 2020). Until a few decades ago, the wild Canarian Egyptian vulture (*Neophron percnopterus var. majorensis*), a subspecies of Egyptian vulture, with a small population inhabited practically in all Canary Islands (Spain), although currently it is only preserved in specific areas of Lanzarote and Fuerteventura Islands. The vultures' population in the Canary Islands has increased by 12% during 2018 (311 individuals recorded in 2017 to 349 individuals) thanks to protection programs promoted by the Regional Government Ministry of Territorial Policy, Sustainability and Security within the European Program Life Egyptian Vulture 2019 (Life Egyptian Vulture (LEV), 2019). According to these same authors, the vultures' population included 361 animals at the end of 2019 (with an increase of 12 vultures, corresponded to 3.4%), from which 163 were classified as reproductive and 198 as non-reproductive animals.

To our knowledge, this is the first report related to ESBL-producing *E. coli* done in Canarian Egyptian vultures. Even though antibiotic resistance in vultures has been less studied than in other domestic animals worldwide (Ahmed *et al*., 2010; Tuerena *et al*., 2016; Carvalho *et al*., 2017; Saputra *et al*., 2017; Rumi *et al*., 2019; Suay-Garcia *et al*., 2019), there are some recent available data in different Spanish cities that suggest that *Neophron percnopterus* might have an important role in the spread of ESBL/pAmpC bacteria (Alcala *et al*., 2016; Lopez-Cerero *et al*., 2017; Moreno-Opo *et al*., 2020; Sevilla *et al*., 2020). In this way, contact with urban waste and livestock farming has been associated with increased probability of antibiotic-resistant microbiota in wild birds, especially ESBL-producing *E. coli*. The aim of this study was to determine the carriage rate of ESBL-producing *E. coli* as well as their genetic characteristics in samples obtained from Canarian Egyptian vultures (*Neophron percnopterus var. majorensis*) from Fuerteventura and Lanzarote (Canary Islands), in Spain. Furthermore, One Health aspects of antimicrobial resistance will be investigated.

Results and discussion

Five of the 22 faecal samples tested carried cefotaxime-resistant (CTX^R) *E. coli* isolates, and were ESBL-producers, representing 22.7% of total samples tested. A similar prevalence of ESBL-producing *E. coli* was found in studies done with black vultures from Mongolia (27%) (Guenther *et al*., 2013) and from three different vulture' species in central Spain (26%) (Lopez-Cerero *et al*., 2017). In the same line, Pinto *et al*. (2010) found a similar prevalence of ESBL-positive isolates among wild birds of prey at the *Serra da Estrela* Natural Reserve in Portugal (26.9%). In contrast, Mora *et al*. (2014) did not found any ESBL-producing *E. coli* between griffon vultures from Southeast of Spain.

Four of our ESBL-producing *E. coli* isolates were obtained from Fuerteventura and only one from Lanzarote Island **(**Table 1). All of them exhibited a multidrug-resistant phenotype (Table 1), which is in accordance with the results obtained with Egyptian vultures in other regions of the Spanish peninsula (Lopez-Cerero *et al*., 2017). It is interesting to note that the ESBL-producing

Table 1. Phenotype and genotype of antimicrobial resistance of the five ESBL-producer *E. coli* isolates recovered from vultures in the Canary Islands.

Isolate number	Origin[a]	Antimicrobial resistance phenotype[b]	β-lactamase genes	Other genes	PG[c]	MLST
X1888	FV	AMP, CTX, TET, CN	CTX-M-15, TEM		B1	ST515
X1889	FV	AMP, AMC, FOX, CTX, CAZ, TET, SXT, CHF, CIP	CTX-M-15, TEM	*int*1	A	ST1290
X1890	LZ	AMP, CTX, CAZ, SXT, TET	CTX-M-15, TEM-1	*tet*A, *int*1	D	ST38
X1891	FV	AMP, CTX, CAZ, SXT, TET, CHF, CIP	CTX-M-55	*int*1	B1	ST6448
X1927	FV	AMP, CTX, CAZ	CTX-M-55		D	ST457

[a]FV: Fuerteventura; LZ: Lanzarote.
[b]AMP: ampicillin; AMC: amoxicillin + clavulanic acid; FOX: cefoxitin; CTX: cefotaxime; CAZ: ceftazidime; TET: tetracycline; SXT: trimethoprim-sulphamethoxazole; CN: gentamicin; S: streptomycin; ERT: ertapenem; CHF: chloramphenicol; CIP: ciprofloxacin.
[c]PG: Phylogenetic group.

isolates found in this study might have an impact on public health if transmitted to humans (Bessalah *et al*., 2016; Grzywaczewski *et al*., 2016). Recently, Sharma *et al*. (2018) found a high rate of beta-lactam resistance among *E. coli* strains in migrant Egyptian vultures in Iran. None of our isolates showed resistance to imipenem (Table 1).

Although the migratory behaviour of raptors has been proposed as a mechanism for the dissemination of antimicrobial-resistant genes (Wang *et al*., 2017), our study is focused on Canarian Egyptian vulture that is not migratory and inhabits only in two islands (Fuerteventura and Lanzarote) of Canary Islands Archipelago. Therefore, only the feeding habits linked to the consumption of remains from intensive livestock farming, especially pigs (Blanco *et al*., 2019) as well as on garbage that accumulate in rubbish dumps (Tauler-Ametller *et al*., 2018) has been associated with a higher probability of antibiotic-resistant microbiota in wild vultures, especially ESBL-producing *E. coli*.

All our isolates carried $bla_{\text{CTX-M}}$ genes, specifically: $bla_{\text{CTX-M-15}}$ (three isolates) and $bla_{\text{CTX-M-55}}$ (two isolates), illustrated in Table 1. All CTX-M-15-producing isolates contained the bla_{TEM} gene and one carried the *tet* (A) gene. Furthermore, three isolates contained the *int*1 gene (Table 1). Pinto *et al*. (2010) detected a high prevalence of CTX-M-1 in ESBL-positive faecal *E. coli* isolates recovered from birds of prey (87.5%; $n = 28/32$) at the *Serra da Estrela* Natural Reserve (Portugal); this ESBL type (CTX-M-1) was also previously reported in studies done by our research group in healthy pets (Costa *et al*., 2004) and wild animals (Costa *et al*., 2006; Poeta *et al*., 2008). Furthermore, Costa *et al*. (2006) detected ESBL producers in 35.7% of the birds of prey studied (5/14 animals), namely CTX-M-1, CTX-M-14 and SHV-12. In Spain, Alcala *et al*. (2016) observed the presence of $bla_{\text{CTX-M-1}}$ gene in a griffon vulture and Lopez-Cerero *et al*. (2017) found the same gene among Egyptian vultures (*Neophron percnopterus*) samples. Other types of beta-lactamases were detected by Batalha-de-Jesus *et al*. (2019) among vultures' samples from Brazil: CTX-M-2, CTX-M-15 and CTX-M-8. Regarding the black vultures isolates from Mongolia, the CTX-M-9 was the most prevalent ESBL detected (Guenther *et al*., 2013).

None of our *E. coli* isolates carried the bla_{SHV} or $bla_{\text{OXA-48}}$ genes. The *mcr*-1 determinant, encoding colistin resistance, was studied in all *E. coli* isolates of this work, and all of them were *mcr*-1 negative. However, Oteo *et al*. (2018) found this gene in one black vulture in Spain. In addition, the focus on chicks might have decreased the frequency of antimicrobial-resistance genes found in their microbiome since such immature birds would be less likely than adult birds to full exposure to environmental antimicrobial resistance genes.

High genetic diversity was observed among the five ESBL *E. coli* isolates with five different sequence types (STs) and three different phylogenetic groups (ST/phylogenetic-group/ESBL): ST515/B_1/CTX-M-15, ST1290/A/CTX-M-15, ST38/D/CTX-M-15, ST457/D/CTX-M-55 and ST6448/B_1/CTX-M-55; this suggests a genetic diversity among these isolates including several strains of STs frequently detected among human clinical isolates. Nevertheless, the lineage ST131 widely disseminated in humans was not detected in this study. These results are in line with another Spanish previous study performed in *E. coli* from healthy dogs showing the detection of ST38 and ST457 (Flament-Simon *et al*., 2019). It is important to note that ST457 *E. coli* is a recent clone with the capacity to cause antimicrobial-resistant extraintestinal infection, almost certainly in dogs, wild animals and possibly in humans (Nicolas-Chanoine *et al*., 2014). Agreeing with our results, a study done by Batalha-de-Jesus *et al*. (2019) showed the presence of CTX-M-2/ST457 in wild birds from Brazil. The ST38 is considered a globally dispersed high-risk clone of extraintestinal pathogenic *E. coli* commonly associated with urinary tract infections and bacteremia in humans (Hernandez *et al*., 2013; Dolejska and Literak, 2019; Shnaiderman-Torban *et al*., 2020) but also found in wildlife (Alcala *et al*., 2016; Guenther *et al*., 2017). According to a recent study, the ST38/CTX-M-15-resistant clone was found in a Mongolian wild

bird (Guenther *et al*., 2017) and the same combination was detected in a human clinical isolate in India (Guiral *et al*., 2019). These results are in line with the fact that ST38 lineage is considered an expanded and pandemic clone as well as this lineage appears to be independent of antimicrobial selective pressure in clinical environments (Guenther *et al*., 2017). The ST515 lineage, also found in our study, has been previously found by Dandachi *et al*. (2018) in poultry from Lebanon, harbouring the *mcr*-1 gene. The same variant was identified in an *E. coli* clinical isolate obtained from a patient in a Canadian hospital (Walkty *et al*., 2016).

Few reports have been done concerning the prevalence and characterization of ESBL-producer *E. coli* in the faecal microbiota of healthy vultures, and most of the studies have focused on *E. coli* isolates from domestic animals (Stolle *et al*., 2013; Ewers *et al*., 2014; Marques *et al*., 2016; Pulss *et al*., 2018) or in wild birds or vultures only from Spanish peninsula (Alcala *et al*., 2016; Lopez-Cerero *et al*., 2017; Moreno-Opo *et al*., 2020). To our knowledge, this is the first study on the genetic background in commensal *E. coli* isolates recovered from Canarian Egyptian vultures in the Canary Islands.

However, we cannot exclude the possibility that these wild animals had been exposed to faecal material of farm animals or even that of humans. These facts might be involved in the acquisition and dissemination of antibiotic-resistant bacteria even in the absence of direct antibiotic pressure and might explain the presence of ESBL-producing *E. coli* isolates. The results of our study support the idea that raptors can act as carriers and spreaders of ESBL-producing *E. coli* representing a risk not only for wildlife but also for livestock and the human population. It is important to note the risk of ESBL-producing *E. coli* for the professionals that work with wild animals, including vultures (Grzywaczewski *et al*., 2016).

Experimental procedures

Animals and sampling

A total of 22 faecal samples from apparently healthy chicks of wild Canarian Egyptian vultures (*Neophron percnopterus var. majorensis*) from Lanzarote ($n = 1$) and Fuerteventura ($n = 21$) were obtained during July 2019 (Fig. 1). All the faecal samples were collected individually using a sterile cotton swab from each animal and were obtained in collaboration with researchers of a long-term monitoring program of vulture population and were processed at the Research Institute of Biomedical and Health Sciences (University of Las Palmas de Gran Canaria, Spain). None of the animals had been fed previously by humans or had received antibiotics. The *Neophron percnopterus var. majorensis* is only distributed in Canary Island territory and it is not a migratory species.

Chicks were captured during the fledgling stage in nests. All procedures, including the capture and handling 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.

Escherichia coli *isolation*

The faecal samples were inoculated onto MacConkey agar plates supplemented with 2 μg ml^{-1} of cefotaxime (MC + CTX) for cefotaxime resistant (CTX^R) *E. coli* recovery. After incubation at 37°C for 24 h, colonies showing *E. coli* morphology were recovered and identified by classical biochemical methods named IMViC (Indol, Methyl-red, Voges–Proskauer and Citrate) and Analytical Profile Index (API 20Egallery). The identification was confirmed with the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry method (MALDI-TOF MS, Bruker) in the Laboratory of Biochemistry and Molecular

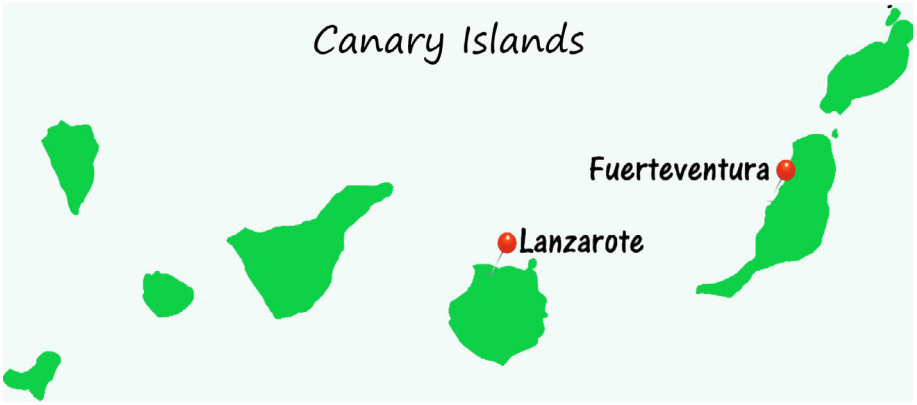

Fig. 1. Origin of vultures' samples: Fuerteventura and Lanzarote (Canary Islands, Spain).

Biology in the *University of La Rioja* (Logroño, Spain). One *E. coli* per sample was kept and characterized by genetic methods.

Susceptibility testing

Antimicrobial susceptibility testing was performed by the agar disk diffusion method, as recommended by the Clinical Laboratory Standards Institute standard guidelines (CLSI, 2018). *Escherichia coli* isolates were tested against the following antimicrobial agents (μg/disk): ampicillin (10), amoxicillin + clavulanic acid (20 + 10), cefotaxime (30), cefoxitin (30), ceftazidime (30), aztreonam (30), imipenem (10), tetracycline (30), nalidixic acid (30), ciprofloxacin (5), trimethoprim-sulfamethoxazole (1.25 + 23.75), gentamicin (10), tobramycin (10), streptomycin (10), amikacin (30) and chloramphenicol (30). Isolates were recorded as susceptible, intermediate, or resistant according to the interpretative standards of zone diameter (CLSI, 2018). The detection of ESBL production was carried out using three disks of antibiotics in the same line: cefotaxime, ceftazidime and amoxicillin/clavulanic acid (CLSI, 2018).

DNA extraction and quantification

Genomic DNA from ESBL-producing isolates was extracted using the InstaGene Matrix (BioRad), according to the manufacturer's instructions. In order to quantify the DNA concentration and the level of purity, the absorbance readings were taken at 260 and 280 nm (Spectrophotometer ND-100, Nanodrop).

Antibiotic resistance genes

The genetic basis of resistance was investigated using PCR and sequencing of the obtained amplicons. Positive controls of the University of La Rioja (Logroño, Spain) were used in this study.

The presence of the beta-lactamase genes [$bla_{\text{CTX-M}}$ (groups 1 and 9), bla_{SHV}, bla_{TEM}, $bla_{\text{OXA-1}}$, and $bla_{\text{OXA-48}}$] was studied by PCR and sequencing (Ruiz *et al*., 2012; Hassen *et al*., 2019). Furthermore, the *mcr*-1 (colistin resistance), *tet*A/*tet*B (tetracycline resistance) and *int*1 genes (integrase of class 1 integrons) were also analysed (Hassen *et al*., 2019). Virulence factors were also tested ($sxt_{1,2}$) (Alonso *et al*., 2017).

The *E. coli* isolates were assigned according to the phylogenetic classification into one of the four main phylogenetic groups, A, B_1, B_2 and D, following a PCR strategy published previously based on the presence or absence of the *chu*A and *yja*A gene or the DNA fragment TSPE4.C2 (Clermont *et al*., 2000).

Multilocus sequence typing of E. coli *strains*

The MLST with seven housekeeping genes (*icd*, *fum*C, *mdh*, *adk*, *rec*A, *pur*A and *gyr*B) was carried out in the five ESBL-positive isolates, according to the PubMLST protocol for *E. coli* (PubMLST, 2020). The allele combination was determined after sequencing the seven genes, and the ST and clonal complex were identified.

Conclusions

Wild birds can contribute to the global spread of ESBL-producing *E. coli* in natural ecosystems. This study shows, for the first time in the Canary Islands, that vultures can be carriers of ESBL-producing *E. coli* isolates associated with diverse STs (ST515, ST1290, ST38, ST457 and ST6448). Our study reports the dissemination of $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-55}}$ genes in *E. coli* isolates from vultures in the Canary Islands. This study supports the hypothesis regarding the circulation of antimicrobial-resistance genes and antimicrobial-resistant bacteria between animals (in this case vultures), humans and the environment.

However, more studies focusing on the human–animal–environmental interface should be performed to better understand the role of these animals in the spread of this type of resistance and to assess potential risks for the public health from the 'One Health' approach.

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