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Research article

Analysis of Eurasian Stone curlew (*Burhinus oedicnemus*) microbial flora reveals the presence of multi-drug resistant pathogens in agro-pastoral areas of Sicily (Italy)

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ABSTRACT

Spread of multi-drug resistant (MDR) bacteria in natural environments pose a risk to human and animal health. Wild birds are considered to be reservoirs of human pathogens and vectors of antimicrobial resistance distribution in the environment. The aim of this study is to assess the occurrence of antibiotic resistant bacteria in isolates from bird specimens living in three agro-pastoral areas of the southeastern Sicily. We analyzed the microbiomes of the Eurasian Stone curlew *Burhinus oedicnemus* (Charadriiformes, Aves) and identified 91 Gram positive and 212 Gram negative strains, whose antimicrobial susceptibility to 11 and 9 antibiotic classes (respectively) was evaluated using agar disk diffusion test. Isolates showed significant levels of antimicrobial resistance, and a high percentage of MDR strains was found both between the Gram positive (49.4%) and the Gram negative (34.9%). Multi-drug resistance levels are higher among strains isolated in the beak and the eye than among enteric (faeces and cloaca) strains. Our results indicate high levels of MDR strains among wild bird populations, with a potential threat to wildlife and human populations.

1. Introduction

The emergence of multiresistant bacteria in natural environments represents a potential hazard to human and animal health (Benskin et al., 2009; Allen et al., 2010; Radhouani et al., 2014). In recent years, substantial evidence has been provided linking high prevalence of antibiotic resistant bacterial strains in the environment with anthropogenic sources (Wellington et al., 2013; Atterby et al., 2016). The disproportionate administration of antibiotics to cattle and other livestock leads to their excretion into the environment in active forms (Berger et al., 1986; Gomes et al., 2017). The exposure of soil microorganisms to these molecules contributes to the development and amplification of resistance, with soil bacteria serving as reservoirs (Rysz and Alvarez, 2004). But effects on wildlife are less investigated, and the extent to which wild populations contribute to the spread of antibiotic resistance is still unknown. Given the increasing number of multidrug-resistant strains (resistant to three or more antibiotic classes), it is of crucial importance to understand the origins of antibiotic resistance in wild bird microbiome. Wild birds have rare contact with antimicrobial agents, thus they could serve as reservoir and potential spreaders of resistant bacteria (Guenther et al., 2010; Järhult et al., 2013; Bonnedahl and Järhult, 2014) and naturally evolving resistance genes (Janatova et al., 2014). This phenomenon is taking place on a global scale, and birds infected with multidrug-resistant bacteria can be found even in environments as remote from intensive farms as the Arctic (Benskin et al., 2009).

Several studies have evidenced the transmission of antibiotic resistant strains, belonging to the same clonal complex, between wildlife or livestock and humans. Conjugation and transfer of R plasmids occurs frequently in the environment, even between unrelated human and animal bacterial strains and in the absence of antibiotics (Kruse and Sorum,

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1994). But our current knowledge of transmission routes is still insufficient, and more studies are necessary in order to evaluate the spread of antibiotic resistance genes (Arnold et al., 2016; Vittecoq et al., 2016).

Resistant bacteria of human and animal origin are likely to be transmitted to wild birds by contaminated food or water (Abulreesh et al., 2007; Bonnedahl et al., 2009; Guenther et al., 2010; Radhouani et al., 2012, 2014; Graham et al., 2014). Moreover, production of natural antibiotics by some bacteria and fungi (Keen and Montforts, 2012) occur naturally throughout the environment. The mixing of environmental bacteria with exogenous bacteria from direct deposition of faeces or urine, use of manure (Heuer et al., 2011) or of effluent flows (Wellington et al., 2013) provides the ideal selective and ecological conditions for new resistant strains to arise.

Many birds use faecal pellets as food resource, searching for invertebrates or worms and ingesting the antibiotics used for the livestock management as well. The few studies that identified potential sources of AMR and made comparisons across sites differing in contamination provide insights into the potential for wildlife to spread clinically relevant AMR. In order to clarify the role of the environment and of wildlife in the antibiotic resistance phenomenon emergence, more studies that analyze the complex ecology and the feedback between human and animal populations and the environment are needed.

Our study was conducted on the Eurasian Stone curlew *Burhinus oedicnemus* (Linnaeus, 1758), a palearctic species belonging to Charadriiformes that is of great conservation interest (Batten et al., 2013). *B. oedicnemus* is a Least Concern species in the Mediterranean and Macaronesian regions (BirdLife International, 2018), but in Italy its mating habitat is rapidly disappearing, and this bird is therefore classified as Vulnerable (Peronace et al., 2012; BirdLife International, 2018). This species nests on the ground, in open canopies characterized by sparse herbaceous vegetation (uncultivated drylands, grazed or halophilous pastures, river groves, etc.) and arboreal plantations (Hume and Kirwan, 2013). Farming practices have a major impact on *B. oedicnemus*' diet, as birds nesting in agro-pastoral areas mainly feed on invertebrates present in ruminant droppings (Spena et al., 2011).

Because of the strong relation between diet and microbiome composition (Brittingham et al., 1988), *B. oedicnemus* can be considered as an indicator of environmental contamination by multiresistant and potentially pathogenic bacteria deriving from human activities, especially farming.

The aim of this study is to assess the levels of antibiotic resistant bacteria in isolates from Eurasian Stone curlew nesting near human settlements in order to evaluate the spread of the antibiotic resistance in wild populations.

2. Materials and methods

2.1. Sampling sites

The samples were taken between July and August 2018 in a large area of the southeastern part of Sicily (Figure 1) in zones characterized by an agro-pastoral environment: the Gela Plain (Caltanissetta), the agro-ecosystems around Ragusa and the Thapsos Peninsula (Siracusa).

These areas host the most relevant nesting and wintering nuclei at regional level (Mascara and Sarà 2007; Tinarelli et al., 2008). The Gela Plain hosts the largest populations of the considered species and the number of pairs is estimated to be 150–200 (Tinarelli et al., 2008). Around Ragusa, the vegetation belongs to the alliance of *Oleo sylvestris-Ceratonion siliquae* Braun-Blanquet, and in the Thapsos Peninsula, carbonate bedrocks are covered by *Thymbra capitata* (L.) Cav. and subnitrophilous vegetation (Spena et al., 2011). In all of the three areas shown in Figure 1, cattle and, to a lesser extent, sheep and goats are present and their droppings represent a valuable food resource for the arthropod-rich fauna (Amat, 1986; Caccamo et al., 2011). The choice of study areas was also made on the basis of the consideration that in heavily populated areas, high background AMR levels often cloud

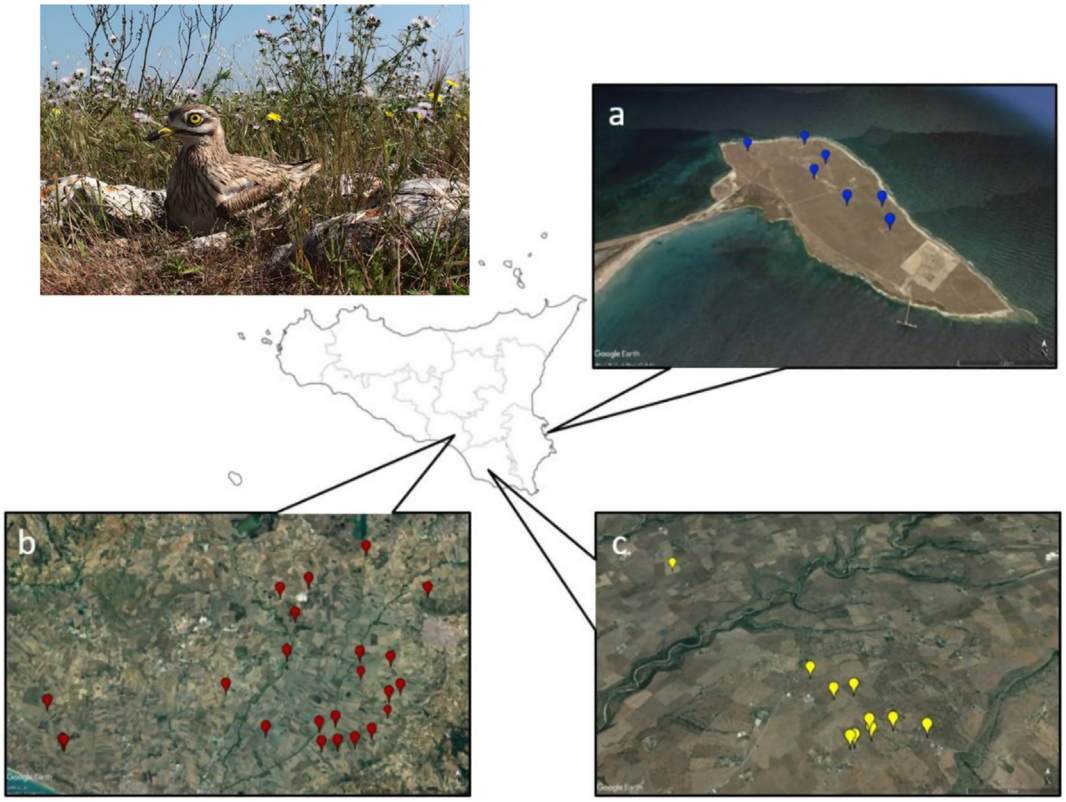

Figure 1. Map showing the investigated areas in southern Sicily (Italy): a) Thapsos Peninsula (Siracusa), b) Gela Plain (Caltanissetta), and c) Ragusa. The coloured drops represent the sampled nests of the *Burhinus oedicnemus*. Photo credit of *Burhinus oedicnemus* from Thapsos Peninsula by MT Spena.

observations while study sites with relatively low or well-defined AMR inputs, as in our case, enable us to quantify spatial patterns, pathways and processes that drive AMR dissemination (Arnold et al., 2016).

The individuals were captured from ground nests using a fall trap and cloacal, conjunctival and oral swabs for bacteriological survey were collected from each bird. Furthermore, whenever possible, a fresh faecal sample was taken. All birds were released immediately after sampling and returned to their nests. Our data indicate that nest disturbance was well tolerated by the birds. Indeed, all continued to incubate after being trapped (Spena et al., 2020).

2.2. Antimicrobial susceptibility testing

The samples from 61 *Burhinus oedicnemus* specimens were previously examined by bacteriological test for commensal and potentially pathogenic bacteria (Spena et al., 2020). The analysis had revealed the presence of 212 Gram negative and 91 Gram positive strains (Tables 1 and 2) (Spena et al., 2020).

We evaluated the antimicrobial susceptibility of all strains isolated (**Supplementary data**). The test was performed by the Disk diffusion method (Bauer et al., 1966) on Mueller-Hinton agar (Oxoid, Basingstoke, UK) in accordance to international standards (CLSI, 2017).

For Gram positive strains susceptibility to 20 antimicrobial agents belonging to 9 antibiotics classes was evaluated: Aminoglycosides [gentamicin (10 μg), tobramycin (10 μg)]; Carbapenems [imipenem (10 μg), meropenem (10 μg)]; Cephalosporins [ceftazidime (30 μg), cefovecin (10 μg), cefepime (30 μg), ceftaroline (30 μg)]; Fluoroquinolones [enrofloxacin (5 μg)]; .Glycopeptides [vancomycin (30 μg)]; Lincosamides [lincomycin (15 μg)]; Macrolides [erythromycin (15 μg)]; Penicillins [amoxicillin (30 μg), ampicillin (10 μg), oxacillin (1 μg), amoxicillin + clavulanic acid (30 μg), ampicillin + sulbactam (20 μg), ticarcillin + clavulanic acid (85 μg)]; Tetracyclines [minocycline (30 μg), tetracycline (30 μg)] (Liofilchem®, Teramo, IT).

For Gram negative strains susceptibility to 20 antimicrobial agents belonging to 11 antibiotics classes was evaluated: Aminoglycosides [gentamicin (10 μg), streptomycin (10 μg), tobramycin (10 μg)]; Carbapenems [imipenem (10 μg), meropenem (10 μg)]; Cephalosporins [cefotaxime (30 μg), cefotaxime + clavulanic acid (40 μg), ceftazidime (30 μg)]; Chloramphenicol [chloramphenicol (30 μg)]; Fluoroquinolones [ciprofloxacin (5 μg), enrofloxacin (5 μg)]; Monobactams [aztreonam (30 μg)]; Penicillins [amoxicillin (30 μg), amoxicillin + clavulanic acid (30 μg), ampicillin (10 μg)]; Polymyxins [colistin sulfate (10 μg)]; Quinolones [nalidixic acid (30 μg)]; Sulfonamides [co-trimoxazole (25 μg)]; Tetracyclines [minocycline (30 μg), tetracycline (30 μg)] (Liofilchem®, Teramo, IT).

The tests were interpreted by measuring the inhibition halo diameter of microorganism growth after incubation at 37 °C for 24 h. Isolates were considered either resistant or susceptible according to the manufacturer's instructions (Liofilchem®, 2019) based on CLSI guidelines (2017), in the case of Polymyxins according to Gales et al. (2001) and in the case of Lincosamides according to Rossney et al. (2007) (Tables 3 and 4). Isolates showing intermediate sensitivity were considered as resistant. Moreover, strains were considered multidrug resistant (MDR) when showing resistance to three or more antimicrobial classes (Magiorakos et al., 2012).

Strains of the same bacterial species found in both faeces and cloacal samples and exhibiting the same resistance profile were considered to be equivalent and therefore only counted once.

2.3. Statistical analysis

As the genera *Bacillus* and *Staphylococcus* have been found to be the most frequently representative Gram positive in our samplings, and *Enterobacter* and *Citrobacter* the most representative among Gram negative, we focused our analysis of the percentage of resistance of the isolates to these strains. Using R-3.6.0, we performed a t-test to determine if a significant difference exists between the means of two groups of microbiomes examined (more specifically beak vs. eye for *Bacillus,*

Table 1. List of tested Gram positive isolated from conjunctival swabs (C) and oral swabs (O).

Bacterial Family	Bacterial species	Number of isolates		
		C	O	Total
Bacillaceae	*Bacillus brevis*	1	1	**2**
	Bacillus cereus subsp *mycoides*		1	**1**
	Bacillus fastidiosus	1		**1**
	Bacillus licheniformis	23	16	**39**
	Bacillus megaterium	5		**5**
	Bacillus pumilus	1		**1**
	Bacillus spp	1		**1**
	Bacillus subtilis		2	**2**
	Exiguobacterium acetylicum		1	**1**
Enterococcaceae	*Enterococcus faecalis*	1	3	**4**
	Enterococcus faecium	2		**2**
Lactobacillaceae	*Lactobacillus rhamnosus*		1	**1**
Paenibacillaceae	*Paenibacillus durus*	1		**1**
Staphylococcaceae	*Staphylococcus aureus*	1	2	**3**
	Staphylococcus cohnii ssp *cohnii*		1	**1**
	Staphylococcus epidermidis		1	**1**
	Staphylococcus gallinarum	1	1	**2**
	Staphylococcus hominis	2	1	**3**
	Staphylococcus lentus	1		**1**
	Staphylococcus saprophyticus		1	**1**
	Staphylococcus sciuri	4	11	**15**
	Staphylococcus warneri	1	1	**2**
	Staphylococcus xylosus		1	**1**
Total		**46**	**45**	**91**

Table 2. List of tested Gram negative isolated from fecal samples (F), cloacal swabs (Cl), conjunctival swabs (C) and oral swabs (O).

Bacterial Family	Bacterial species	Number of isolates				
		F	Cl	C	O	Total
Aeromonadaceae	*Aeromonas sobria*				1	**1**
	Aeromonas hydrophila				1	**1**
Enterobacteriaceae Group	*Citrobacter amalonaticus*	5	4			**9**
	Citrobacter diversus			4	3	**7**
	Citrobacter farmeri	1	2			**3**
	Citrobacter freundii	3	3			**6**
	Citrobacter spp	13	18		1	**32**
	Enterobacter aerogenes	2	6		1	**9**
	Enterobacter asburiae			1	1	**2**
	Enterobacter cancerogenus	2	1		2	**5**
	Enterobacter cloacae	6	4	8	15	**33**
	Enterobacter kobei		3		2	**5**
	Enterobacter ludwigii		2			**2**
	Enterobacter spp				1	**1**
	Escherichia coli	14	5	4	2	**25**
	Escherichia hermannii				1	**1**
	Hafnia alvei	6	2	3	2	**13**
	Kluyvera ascorbata	1				**1**
	Leclercia adecarboxylata	1		8	3	**12**
	Proteus mirabilis	2	5			**7**
	Proteus vulgaris			1	1	**2**
	Providencia rettgeri		2			**2**
	Salmonella enterica ssp *enterica*	4				**4**
	Serratia liquefaciens	1				**1**
	Serratia rubidaea				1	**1**
	Shigella dysenteriae		1			**1**
Flavobacteriaceae	*Chryseobacterium indologenes*			3	1	**4**
Pseudomonadaceae	*Pseudomonas aeruginosa*	2	3		1	**6**
	Pseudomonas putida				1	**1**
	Pseudomonas stutzeri		1	7	5	**13**
Xanthomonadaceae	*Stenotrophomonas maltophilia*				1	**1**
Vibrionaceae	*Vibrio mimicus*	1				**1**
	Total	**64**	**62**	**39**	**47**	**212**

Staphylococcus and *Enterobacter* spp. and faeces vs. cloaca for *Citrobacter* and *Enterobacter* spp.). We fixed the significance limit for the statistical analyses at $P = 0.05$. In addition, we used a 2-sample Kolmogorov-Smirnov test to evaluate the difference in AMR levels distribution between different isolates, using the R-3.6.0 ks.test function.

Finally, we evaluated the extent to antimicrobial resistance levels are related to direct contact with environmental pathogens. In order to do this, we compared the resistance levels recorded in Gram negative strains likely originating from direct contact with the environment (isolated from eye and beak) with those recorded in enteric Gram negative strains isolated from faeces and cloaca using Fisher's Exact Test, fixing the significance limit at $P = 0.05$.

3. Results

The overall resistance to antibiotics of all Gram positive and Gram negative isolates points out a high resistance to some of tested molecules. The resistance patterns varied from one to eighteen in Gram positive and from one to twelve in Gram negative strains.

Gram positive strains often exhibited high values of resistance (Table 5), and multidrug resistance occurred in 45 strains (49.4%) (**Supplementary data**).

The majority of the Gram positive isolates were resistant to ceftazidime (90.1%) and oxacillin (81.3%). Very low levels of resistance were found against Tetracyclines and Carbapenems (from respectively 2,2% to and 4.4%). Seven Gram positive strains (7.7%) were susceptible to all tested antibiotics. This analysis only included 207 of the 212 strains, as 5 strains of faecal origin presented an identical resistance profile to that of cloacal strains isolated from the same individual. They were therefore considered equivalent and only counted once.

Some Gram negative isolates displayed a wide antibiotic resistance (Table 6) and multidrug resistance occurred in 74 strains (35.7%) (**Supplementary data**).

The majority of the Gram negative isolates were resistant to colistin sulfate (70.7%) and amoxicillin (52.3%). Low levels of resistance (3.1%) were found against Carbapenems also for the Gram negative isolates, but no resistance was found against Fluoroquinolones.

The most abundant MDR pattern observed in Gram negative was combined resistance to Aminoglycosides, Penicillins and Polymyxins while in Gram positive was combined resistance to Cephalosporins, Penicillins and Lincosamides.

Comparing the resistance percentages based on the sample, the highest values were found in the beak and eye isolates. Figure 2 shows the record distribution of the 177 strains according to the resistance to antibiotics of either the Gram positive or the Gram negative bacteria.

Gram negative strains isolated from the eye and the beak display considerably higher levels of resistance compared to enteric Gram negative (isolated from faeces and cloacal swabs). This difference is

Table 3. List of antibiotics used in Gram positive antimicrobial susceptibility tests and relative inhibition halos diameters for resistant (R), intermediate (I) and sensitive (S) strains.

Class	Antibiotics	Code µg	Diameter (mm)		
			R <	I	S >
Aminoglycosides	gentamicin	CN 10	12	13–14	15
	tobramycin	TOB 10	12	13–14	15
Carbapenems	imipenem	IMI 10	16	17–21	22
	meropenem	MRP 10	16	17–21	22
Cephalosporins	ceftazidime	CAZ 30	14	15–17	18
	cefovecin	CVN 10	14	15–20	21
	cefepime	FEP 30	14	15–17	18
	ceftaroline	CPT 30	23		23
Fluoroquinolones	enrofloxacin	ENR 5	17	18–20	21
Glycopeptides	vancomycin	VA 30	14	15–16	17
Lincosamides	lincomycin	MY 15	14	15–20	21
Macrolides	erythromycin	E 15	13	14–22	23
Penicillins	amoxicillin	AML 30	13	14–16	17
	amoxicillin + clavulanic acid	AUG 30	19		20
	ampicillin *Staphylococcus* spp.	AMP 10	13 28	14–16	17 29
	ampicillin + sulbactam	AMS 20	11	12–14	15
	oxacillin *Staphylococcus* spp. coagulase -	OX 1	10 18	11–12	13 19
	ticarcillin + clavulanic acid	TIM 85	22		23
Tetracyclines	minocycline	MN 30	14	15–18	19
	tetracycline	TE 30	11	12–14	15

Table 4. List of antibiotics used in Gram negative antimicrobial susceptibility tests and relative inhibition halos diameters for resistant (R), intermediate (I) and sensitive (S) strains.

Class	Antibiotics	Code µg	Diameter (mm)		
			R <	I	S >
Aminoglycosides	gentamicin	CN 10	13	14–15	16
	streptomycin	S 10	11	12–14	15
	tobramycin	TOB 10	13	14–15	16
Carbapenems	imipenem	IMI 10	19	20–22	23
	meropenem	MRP 10	19	20–22	23
Cephalosporins	cefotaxime	CTX 30	22	23–25	26
	cefotaxime + clavulanic acid	CTL 40	22	23–25	26
	ceftazidime	CAZ 30	17	18–20	21
Chloramphenicol	chloramphenicol	C 30	12	13–17	18
Fluoroquinolones	ciprofloxacin	CIP 5	19	20–21	22
	enrofloxacin	ENR 5	15	16–20	21
Monobactams	aztreonam	ATM 30	17	18–20	21
Penicillins	amoxicillin	AML 30	13	14–17	18
	amoxicillin + clavulanic acid	AUG 30	13	14–17	18
	ampicillin	AMP 10	13	14–16	17
Polymyxins	colistin sulfate	CS 10	11	12–16	17
Quinolones	nalidixic acid	NA 30	13	14–18	19
Sulfonamides	co-trimoxazole	SXT 25	13	14–15	16
Tetracyclines	minocycline	MN 30	12	13–15	16
	tetracycline	TE 30	11	12–14	15

highly significant for a range of antibiotics, including gentamicin ($P = 0.007$), ceftazidime ($P = 0.00001$), aztreonam ($P = 0.00009$) and nalidixic acid ($P = 0.0002$) (Table 7).

Within-genera comparison suggests that this difference is caused by the different composition of the enteric and the beak/eye microbiomes. MDR levels also tends to be higher in bacteria found in the beak or eye when compared to enteric bacteria from the same genus, although such differences are not statistically significant (Table 8).

Among the strains treated with antimicrobial classes common for Gram positive and Gram negative bacteria, beak microbiomes show a much higher tolerance against Aminoglycosides, Penicillins and Tetracyclines than eye microbiomes do. The resistance against Cephalosporins show opposite trends for Gram positive and Gram negative bacteria in

Table 5. Numbers (n) and percentages (%) of Resistant Gram positive strains for single molecules (first couple of values) and their classes (second couple of values).

Class		Antibiotics	n. Res	% Res	n. Res	% Res
Aminoglycosides		gentamicin	17	18.7	36/182	19.8
		tobramycin	19	20.9		
Carbapenems		imipenem	4	4.4	8/182	4.4
		meropenem	4	4.4		
Cephalosporins	III generation	ceftazidime	82	90.1	120/182	65.9
		cefovecin	38	41.7		
	IV generation	cefepime	35	38.5	35/91	38.5
	V generation	ceftaroline	15	16.5	15/91	16.5
Fluoroquinolones		enrofloxacin	6	6.6	6/91	6.6
Glycopeptides		vancomycin	17	18.7	17/91	18.7
Lincosamides		lincomycin	32	35.2	32/91	35.2
Macrolides		erythromycin	24	26.4	24/91	26.4
Penicillins	Aminopenicillins	amoxicillin	25	27.5	47/182	25.8
		ampicillin	22	24.2		
	Isoxacillin	oxacillin	74	81.3	74/91	81.3
	Penicillins + β-lactamase inhibitors	amoxicillin + clav ac	7	7.7	16/273	5.9
		ampicillin + sulbactam	6	6.6		
		ticarcillin + clav ac	3	3.3		
Tetracyclines		minocycline	2	2.2	4/182	2.2
		tetracycline	2	2.2		

Table 6. Numbers (n) and percentages (%) of Resistant Gram negative strains for single molecules (first couple of values) and their classes (second couple of values).

Class		Antibiotics	n. res	% res	n. res	% res
Aminoglycosides		gentamicin	13	6.3	107/621	17.2
		streptomycin	73	35.3		
		tobramycin	21	10.1		
Carbapenems		imipenem	10	4.8	13/414	3.1
		meropenem	3	1.4		
Cephalosporins	III generation	ceftazidime	18	8.7	25/414	6
		cefotaxime	7	3.4		
	Cephalosporins + β-lactamase inhibitors	cefotaxime + clav ac	2	1	2/207	1
Fluoroquinolones		ciprofloxacin	-	-	7/414	1.7
		enrofloxacin	7	3.4		
Monobactams		aztreonam	16	7.7	16/207	7.7
Penicillins	Aminopenicillins	amoxicillin	109	52.7	186/414	44.9
		ampicillin	77	37.2		
	Penicillins + β-lactamase inhibitors	amoxicillin + clav ac	95	45.9	95/207	45.9
Phenicols		chloramphenicol	7	3.4	7/207	3.4
Polymyxins		colistin sulfate	147	71	147/207	71
Quinolones		nalidixic acid	20	9.7	20/207	9.7
Sulfonamides		co-trimoxazole	9	4.3	9/207	4.3
Tetracyclines		minocycline	16	7.7	32/414	7.7
		tetracycline	16	7.7		

beak and eye, as the Gram negative resistance is much higher in the beak than in the eye, in contrast to the Gram positive microbiomes where beak and eye bacteria show an almost equal resistance against Cephalosporins (**Supplementary data**).

The minimum levels of AMR found in *Enterobacter* spp. is 10% (Figure 3). A similar value is found among *Staphylococcus* spp. from eye isolates, with a slightly lower value found in beak isolates (Figure 3). *Citrobacter* spp. was recorded only four times in the beak and in the eye, whilst it has been recorded often in fecal and cloacal samples (Figure 3).

Comparison between beak and eye isolates reveals no significant difference in AMR levels for several bacterial genera (*Bacillus*, $t = 0.754$, $df = 50$;*Staphylococcus*, $t = 0.406$, $df = 28$; *Enterobacter*, $t = 1.572$, $df = 27$) (beak and eye). Similarly, there is no significant difference in mean AMR levels between the most common genera of bacteria isolated from the faeces and from the cloaca (*Enterobacter*; $t = 0.463$, $df = 24$; *Citrobacter*, $t = 0.966$, $df = 47$). However, the spectrum of antibiotic resistance differs markedly between isolates (Figures 2 and 3). Kolmogorov-Smirnov (KS) test showed significant differences between the beak and the eye for *Bacillus* ($P = 0.3645$), *Staphylococcus* ($P = 0.978$) and *Enterobacter,* ($P = 0.3504$); and between faeces and cloaca for *Enterobacter* ($P = 0.9998$) and for *Citrobacter* ($P = 0.7154$) (a high p-value in the KS-test indicates a high likelihood that samples come from two distinct distributions).

4. Discussion

Our results evidence the role of wild birds as reservoirs and potential vectors of AMR bacteria. *B. oedicnemus'* feeding habits put it in close

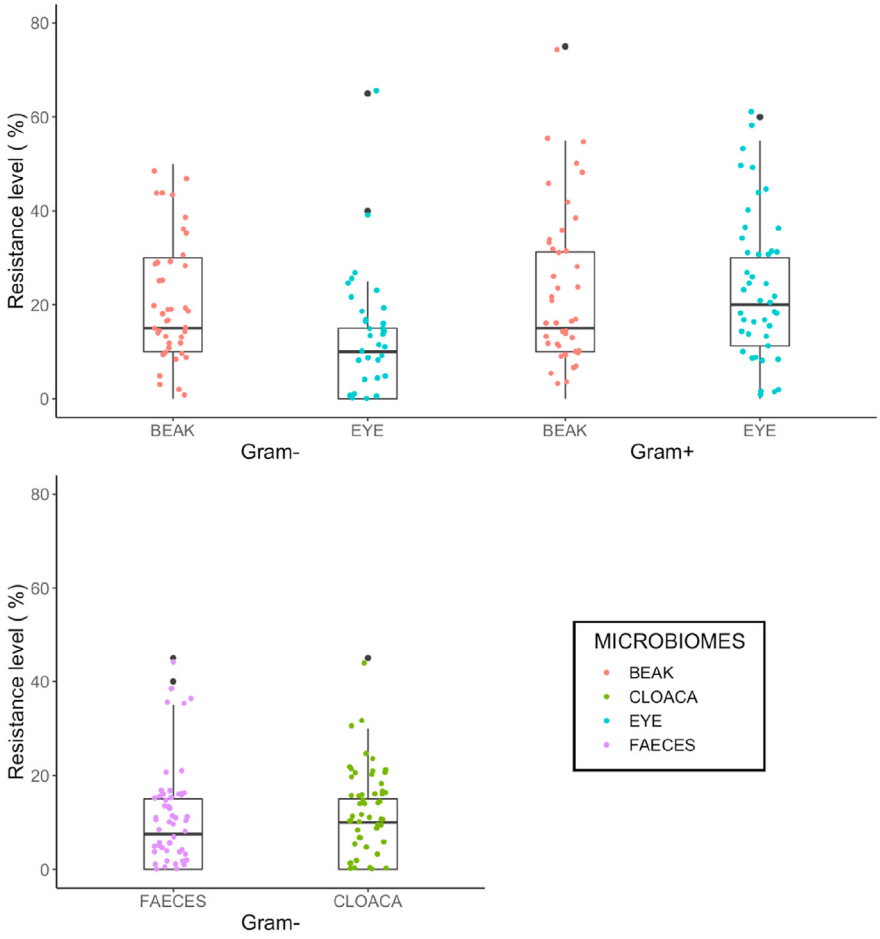

Figure 2. Antibiotic resistance distribution of Gram positive (*left*, $n = 91$) and Gram negative (*right*, $n = 86$) isolates from beak and eye.

Table 7. Comparison of the resistance number recorded in Gram negative strains isolated from fecal samples (F) and cloacal swabs (Cl) (n. 121) vs. Gram negative strains isolated from conjunctival swabs (C) and oral swabs (O) (n. 86) by Fisher's Exact Test.

Antibiotics	Number resistant strains		P Fischer Values
	F + Cl	C + O	
gentamicin	3 (2.5)	10 (11.6)	0.008
streptomycin	37 (30.6)	36 (41.8)	0.08
tobramycin	8 (6.6)	13 (15.1)	0.06
Imipenem	4 (3.3)	6 (7)	0.32
meropenem	0	3 (3.5)	0.07
ceftazidime	2 (1.7)	16 (18.6)	0.00001
cefotaxime	2 (1.7)	5 (5.8)	0.12
cefotaxime + clav ac	0	2 (2.3)	0.16
chloramphenicol	5 (4.1)	2 (2.3)	0.70
ciprofloxacin	-	-	-
enrofloxacin	2 (1.7)	5 (5.8)	0.12
Aztreonam	2 (1.7)	14 (16.3)	0.00009
Amoxicillin	56 (46.3)	53 (61.6)	0.02
Ampicillin	41 (33.9)	36 (41.9)	0.19
amoxicillin + clav ac	50 (41.3)	45 (52.3)	0.09
colistin sulfate	80 (66.1)	67 (77.9)	0.03
nalidixic acid	4 (3.3)	16 (18.6)	0.0002
co-trimoxazole	3 (2.5)	6 (7)	0.16
minocycline	8 (6.6)	8 (9.3)	0.44
tetracycline	10 (8.3)	6 (7)	1.00

( ) = percentage.

Table 8. Number of MDR strains divided into bacterial genera and according to the source sample.

Genera	F + Cl		C + O		P
	n. MDR/n. total strains	%	n. MDR/n. total strains	%	
Aeromonas spp.	0/0	-	1/2	50	-
Citrobacter spp.	10/49	20.4	5/8	62.5	0.13
Chryseobacterium spp.	0/0	-	4/4	100	-
Enterobacter spp.	8/26	30.8	19/31	61.3	0.23
Escherichia spp.	4/19	21.1	1/6	16.7	1.00
Hafnia spp.	0/8	0.00	1/5	20	0.43
Leclercia spp.	0/1	0.00	3/11	27.3	1.00
Proteus spp.	4/7	57.1	1/2	50	1.00
Providencia spp.	1/2	50	0/0	-	-
Pseudomonas spp.	3/6	50	5/14	35.7	1.00
Serratia spp.	1/1	100	1/1	100	1.00
Shigella spp.	1/1	100	0/0	-	-
Stenotrophomonas spp.	0/0	-	1/1	100	-

contact with bacteria from livestock, making it an excellent indicator of environmental contamination. To the best of our knowledge, *B. oedicnemus* is the only significant source of AMR dissemination identified in the areas were the study was conducted.

The range anti-microbial resistance levels in wild birds reported in the literature is broad. Such variations can often be attributed to differences in habitat (Carter et al., 2018), as demonstrated by several studies that measure resistance levels in isolates from birds with different lifestyles or captured in different environments (Shobrak and Abo-Amer, 2014; Atterby et al., 2016; Giacopello et al., 2016; Ramey et al., 2018; Tormoehlen et al., 2019). Our results are consistent with those reported in similar investigations, but strongly differ from other results found in the literature. Atterby et al. (2016) detected lower resistance levels than ours to all tested molecules in birds from a remote location in South-central Alaska, but found higher percentages for Cephalosporins, Quinolones and for Tetracyclines in a more urban setting, with similar levels

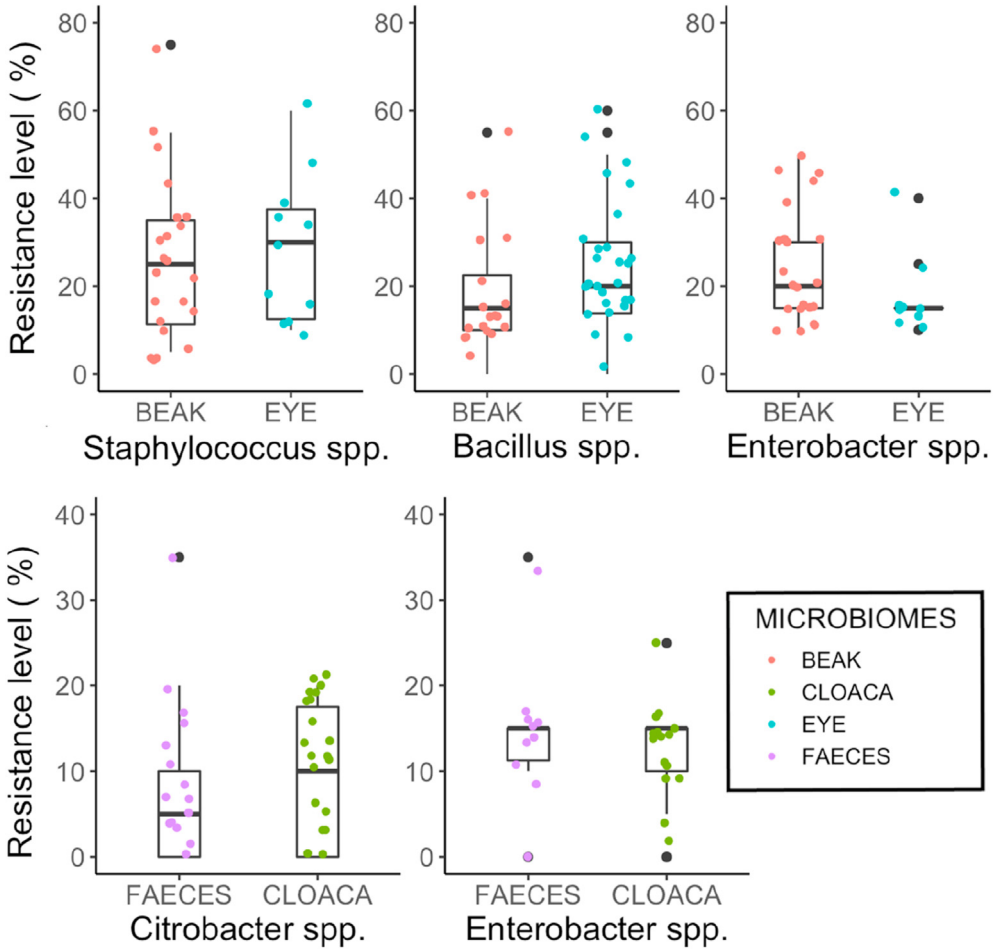

Figure 3. Antibiotic resistance distribution of *Bacillus* and *Staphylococcus* (Gram positive) and *Enterobacter* (Gram negative) in beak and eye (*a*, upper panel) and *Enterobacter* and *Citrobacter* (Gram negative) in faeces and cloaca (*b*, lower panel).

of Aminoglycosid resistance. Shobrak and Abo-Amer (2014) report higher values in both migratory and non-migratory birds, but found no resistance to Aminoglycosid in non-migratory ones. We have, however, detected higher frequency of Polymyxins resistance compared to their values (24% in non-migratory and 37.5% in migratory birds). Botti et al. (2013) found lower percentage values than ours above all for Polymyxins in wildlife of Nord Italy.

Colistin sulfate is typically used as a last resort antibiotic to treat of MDR infections in humans, and has been regularly employed in veterinary medicine both as treatment and a preventative measure of Gram negative gastrointestinal infections. On 27 July 2016, the European Commission issued a Decision (EMA/CVMP/CHMP/231573/2016) which limits the use of colistin in the veterinary field and prohibits its use in combination with other antibiotics (European Commission, 2016). In the coming years, Italy will have to commit itself to reducing consumption within "desirable" values. In this regard, countries such as Italy and Spain, considered "high and moderate consumers", will have to set the goal of reducing consumption from 20-25 mg/population correction unit (currently recorded) to 5 mg/population correction unit of colistin (minimum target level).

Resistance levels against carbapenems and fluoroquinolone, whose use in veterinary medicine is restricted, are low compared to other classes of antibiotics. This supports the hypothesis that contact with livestock is the main cause of AMR spread in wild populations.

Both Gram positive as Gram negative strains display some resistance against Carbapenems. The resistance to a class of antibiotics generally used as an emergency measure against MDR bacteria together with the high resistance to other antibiotic classes, constitutes a potential threat to wildlife and therefore deserves further investigation.

Our results indicate a significant difference in the spectrum of antibiotic resistance between isolates from beak and eye and between faeces and cloaca. This suggests that an evaluation of the microbial flora aimed at obtaining epidemiological information must include sampling carried out on different regions of the body. In particular, our results show that a comprehensive evaluation of the intestinal microbiome must rely not only on cloacal swabs, but also on faecal samples.

5. Conclusion

Evaluation of antibiotic resistance levels in wildlife (especially in wild birds) is often limited to a few pathogenic bacteria such as *Escherichia coli*, *Enterococcus* spp., and *Salmonella* spp. As a consequence, little is known about the broader diversity of antibiotic resistant bacteria in wildlife and the environment (Carter et al., 2018). In our research, we examined 303 strains belonging to 22 genera (11 families) finding AMR in all of them. Many strains belonged to commensal bacterial species of the body sites from which they were isolated (eye, beak, intestine), suggesting the existence of more diffusion routes of resistant strains than the fecal deposition route typically reported in the literature (Guenther et al., 2010; Järhult et al., 2013; Shobrak and Abo-Amer, 2014). Our results show that commensal bacteria play an important role in the transmission of anti-microbial resistance, but further epidemiological studies are necessary in order to gain a more detailed understanding of this complex phenomenon.

Declarations

Author contribution statement

Maria Foti, Rosario Grasso: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Vittorio Fisichella, Antonietta Mascetti, Manuel Andrea Zafarana, Maria Grasso: Performed the experiments.

Marco Colnaghi: Analyzed and interpreted the data; Wrote the paper.

Maria Teresa Spena: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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