DOI: 10.1002/vms3.38

Original Article

Antibiotic resistance in conjunctival and enteric bacterial flora in raptors housed in a zoological garden

Andrea Sala*, Simone Taddei*, Davide Santospirito*, Camillo Sandri†, William Magnone† and Clotilde S. Cabassi*

**Department of Veterinary Science, University of Parma, Via del Taglio 10, 43126, Parma, Italy and †Parco Natura Viva, loc. Figara 40, 37012, Bussolengo, Verona, Italy*

Abstract

Antimicrobial resistance (AMR) in a wide range of infectious agents is a growing public health threat. Birds of prey are considered indicators of the presence of AMR bacteria in their ecosystem because of their predatory behaviour. Only few data are reported in the literature on AMR strains isolated from animals housed in zoos and none about AMR in raptors housed in zoological gardens. This study investigated the antibiotic sensitivity profile of the isolates obtained from the conjunctival and cloacal bacterial flora of 14 healthy birds of prey, 6 *Accipitriformes*, 3 *Falconiformes* and 5 *Strigiformes*, housed in an Italian zoological garden. *Staphylococcus* spp. was isolated from 50% of the conjunctival swabs, with *S. xylosus* as the most common species. From cloacal swabs, *Escherichia coli* was cultured from all animals, while *Klebsiella* spp. and *Proteus* spp. were isolated from a smaller number of birds. Worthy of note is the isolation of *Escherichia fergusonii* and *Serratia odorifera*, rarely isolated from raptors. Staphylococci were also isolated. All the isolates were multidrug resistant (MDR). To the author's knowledge, this is the first report regarding the presence of MDR strains within raptors housed in a zoological garden. Since resistance genes can be transferred to other pathogenic bacteria, this represents a potential hazard for the emergence of new MDR pathogens. In conclusion, the obtained data could be useful for *ex-situ* conservation programmes aimed to preserve the health of the endangered species housed in a zoo.

Keywords: captive raptors, antibiotic resistance, MDR isolates, conjunctival bacteria, enteric bacteria.

Correspondence: Clotilde S. Cabassi, Department of Veterinary Science, University of Parma, Via del Taglio 10, 43126 Parma, Italy.
E-mail: clotildesilvia.cabassi@unipr.it

Introduction

'Antimicrobial resistance (AMR) within a wide range of infectious agents is a growing public health threat…'. These words introduce, in the latest report of the World Health Organization on AMR, one of the major challenges for modern medicine of the 21st century (WHO 2014) involving humans and animals (Shallcross & Davies 2014; Grobbel *et al.* 2007). The isolation of AMR bacteria from wild animals has been reported, including many species of birds of prey (Rouffaer *et al.* 2014). Birds of prey are considered indicators of AMR bacteria in their ecosystem because of their predatory behaviour (Smith *et al.* 2002). In the literature, only a few papers exist on AMR strains isolated from animals housed in zoos (Baldy-Chudzik *et al.* 2008); moreover, in author's knowledge no information is available about AMR in raptors housed in zoological gardens. The dissemination of AMR bacteria in zoos can be favoured by the conservation programmes (*ex-situ* conservation programmes and multi-species exhibits), which are based on the cohabitation among different animal species. Many modern zoos, included in international associations such as World Association of Zoos and Aquaria or European Association of Zoos and Aquaria (EAZA), are involved in a breeding programme based on a network of animal exchanges between the various members (EAZA 2010). These practices may represent a potential route of dissemination of AMR bacterial strains between zoological gardens. Moreover, the possible reintroduction of

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wild animals, or of their offspring, kept in zoos to the wildlife can represent a critical point for the release of AMR bacteria in the environment (Radhouani *et al.* 2012).

The aim of this study was to investigate antibiotic resistance, in particular the multidrug resistance (MDR), among the bacterial strains isolated from cloacal and conjunctival swabs of captive raptors housed in a zoological garden in Italy.

Materials and methods

Samples were collected in autumn (September-October) from 14 birds of prey, 3 *Falconiformes*: 3 mountain caracaras (*Phalcoboenus megalopterus*), 6 *Accipitriformes*: 4 Egyptian vultures (*Neophron percnopterus*) and 2 red-headed vultures (*Sarcogyps calvus*), 5 *Strigiformes*: 2 Eurasian eagle-owls (*Bubo bubo*) and 3 snowy owls (*Bubo scandiacus*), housed in a zoological garden located in northern Italy (Parco Natura Viva - Bussolengo, Verona, Italy). All the animals were healthy, on the basis of a general physical examination performed by the zoo veterinarian, and not previously treated with antibiotics. With the exception of the Egyptian vultures, who shared the cage with storks, all birds were sorted in groups by species and kept in dedicated aviaries, bounded by grid, with a large outdoor area and a shelter area according to the EAZA guidelines (2014) (EAZA 2014). Birds diet was determined depending on bird species and weight. For all birds, the diet was based on alternate-day administration of chicken thighs or turkey wings. Moreover, various dietary supplements, consisting of whole carcasses of rabbits or chicks or quail or rats or mice, depending on raptor species, were regularly provided. Mountain caracaras and red-headed vultures were sometimes also supplemented with fruits. Conjunctival samples were taken from the conjunctival sac of each eye by sterile swabs with Amies transport medium. The enteric flora was assessed by cloacal swabs with Amies transport medium. All samples were chilled at 4°C and submitted to the laboratory of Infectious Diseases of Animals of the University of Parma within 12 h. Each sample was streaked onto tryptose agar (Beckton Dickinson, Sparks, MD, USA) containing 5% bovine erythrocytes and Mc Conkey agar (Beckton) and incubated aerobically for 24–48 h at 37°C. Identification of bacterial isolates was based on their growth and colony characteristics, Gram staining, cellular morphology, catalase and oxidase reactions. Species identification was carried out using the API Staph and API 20 E biochemical test systems (bioMérieux, Marcy-l'Etoile, France), as well as conventional biochemical tests (Quinn *et al.* 1994). Antimicrobial susceptibility tests were performed by Kirby-Bauer disk-diffusion method (Carter *et al.* 1994).

Antibiotic resistance was evaluated on the basis of the criteria proposed by (Magiorakos *et al.* 2012) and adopted by the European Centre for Disease Prevention and Control (ECDC) and the Centre for Disease Control and Prevention (CDC) (ECDC 2012; CDC 2013). An isolate is considered MDR when it is non-susceptible to at least one antibiotic in at least three classes of antimicrobials.

The research complies with the current law of the European Union and Italy regarding the protection of animals used for experimental and other scientific purposes (Directive 86/609/EEC - D. L.vo 116/92 and Directive 2010/63/EU – D. L.vo 26/2014).

Results

Seven out of 14 birds (50%) had positive culture from conjunctival swabs. Bacterial isolates were recovered from 5 out of 5 *Strigiformes*, and from 2 out of 3 *Falconiformes* and belonged to the *Staphylococcus* genus. All conjunctival swabs cultures from *Accipitriformes* were negative. The most common was *S. xylosus* ($n = 4$), while *S. chromogenes, S. lentus* and *S. aureus* were isolated only once (Table 1).

In cloacal swabs, Gram-positive bacteria were isolated from 6 birds (3 *Strigiformes* and 3 *Falconiformes)*, while Gram-negatives were cultured from all birds. The most frequent was *Escherichia coli* ($n = 14$), followed by *Klebsiella* spp. ($n = 7$), *Proteus* spp. ($n = 6$) and *Staphylococcus* spp. ($n = 6$). Isolates of *Klebsiella* and *Proteus* were identified to species level as *Klebsiella pneumoniae* in two cases and *Proteus mirabilis* in one case, while the API test was not able to discriminate the other isolates (Table 1). All

Table 1. Bacteria isolated from conjunctival and cloacal swabs

	Number (%) of animals positive			
	Accipitriformes ($N = 6$)	Falconiformes ($N = 3$)	Strigiformes ($N = 5$)	Total ($N = 14$)
Conjunctival swabs				
Staphylococcus xylosus	0 (0)	0 (0)	4 (80)	4 (29)
Staphylococcus chromogenes	0 (0)	0 (0)	1 (20)	1 (7)
Staphylococcus lentus	0 (0)	1 (33)	0 (0)	1 (7)
Staphylococcus aureus	0 (0)	1 (33)	0 (0)	1 (7)
Cloacal swabs				
Staphylococcus aureus	0 (0)	2 (67)	2 (40)	4 (29)
Staphylococcus simulans	0 (0)	1 (33)	0 (0)	1 (7)
Staphylococcus xylosus	0 (0)	0 (0)	1 (20)	1 (7)
Escherichia coli	6 (100)	3 (100)	5 (100)	14 (100)
Escherichia fergusonii	2 (33)	0 (0)	0 (0)	2 (14)
Proteus spp.	3 (50)	1 (33)	1 (20)	5 (36)
Proteus mirabilis	1 (17)	0 (0)	0 (0)	1 (7)
Klebsiella spp.	3 (50)	0 (0)	2 (40)	5 (36)
Klebsiella pneumoniae	0 (0)	0 (0)	2 (40)	2 (14)
Kluyvera spp.	0 (0)	0 (0)	2 (40)	2 (14)
Serratia odorifera	0 (0)	0 (0)	2 (40)	2 (14)
Citrobacter youngae	1 (17)	0 (0)	0 (0)	1 (7)
Providencia stuartii	0 (0)	1 (33)	0 (0)	1 (7)
Raoultella ornithinolytica	1 (17)	0 (0)	0 (0)	1 (7)

Gram-positives belonged to the *Staphylococcus* genus. Detailed results are reported in Table 1. The antibiotic susceptibility test results on isolates from conjunctival and cloacal swabs are listed in Table 2 and 3, respectively. Antibiotic susceptibility profiles for the following strains, of limited relevance for birds and public health and with lower frequency of isolation, were not reported in Table 3. *Citrobacter youngae* was resistant to tetracyclines, aminoglycosides, macrolides and lincosamides, and to one drug in the penicillins, cephalosporins, cloramphenicol and quinolones classes. *Providencia stuartii* was resistant to all cephalosporins, aminoglycosides, macrolides, lincosamides and penicillins (except amoxicillin/clavulanic acid). *Raoultella ornithinolytica* and *Kluyvera* spp. showed resistance against all drugs in the sulphonamides, penicillins, cephalosporins, tetracyclines, aminoglycosides, macrolides, lincosamides and quinolones classes. At last, *Serratia odorifera* was not susceptible to all the tested antibiotics except amoxicillin/clavulanic acid, cephalexin, cefovecin, enrofloxacin and marbofloxacin. Therefore, all our isolates were MDR.

Discussion

AMR is one of the most serious emerging health threats (Rossolini *et al.* 2014). The nature and extent of this problem are studied extensively in both humans and animals (Shallcross & Davies 2014). Free-living raptors were already investigated as carriers of AMR strains (Rouffaer *et al.* 2014). However, the impact of this phenomenon among birds of prey housed in zoological gardens has not yet been investigated.

We observed a clear predominance of Gram-positive isolates from conjunctival swabs from clinically healthy eyes. In particular, coagulase-negative staphylococci (CoNS) were the most frequent. This finding is in agreement with previous surveys performed on several species of reptiles and birds (Dupont *et al.* 1994; Taddei *et al.* 2010; Di Ianni *et al.* 2015). CoNS are usually non-pathogenic, but occasionally can be involved in ocular infections, influenced by population density, gender or age (Benskin *et al.* 2009). In our samples, *S. aureus* has been isolated from healthy eyes. This known pathogen can

Table 2. Antibiotic resistance of bacterial isolates from conjunctival swabs

Antibiotics		Number (%) of resistant isolates	
		Coagulase-negative *Staphylococcus* spp. ($N = 6$)	*Staphylococcus aureus* ($N = 1$)
Sulphonamides	TS*	3 (50)	1 (100)
Penicillins	PG	5 (83)	1 (100)
	AP	5 (83)	1 (100)
	A	6 (100)	1 (100)
	AMC	6 (100)	1 (100)
Cephalosporins	CDX	0 (0)	0 (0)
	CL	0 (0)	0 (0)
	KZ	6 (100)	1 (100)
	CVN	0 (0)	0 (0)
Tetracyclines	OT	2 (33)	1 (100)
	DXT	6 (100)	1 (100)
Aminoglycosides	S	2 (33)	1 (100)
	K	0 (0)	0 (0)
	GM	6 (100)	1 (100)
	AK	0 (0)	0 (0)
Macrolides	E	3 (50)	1 (100)
	TY	6 (100)	1 (100)
Lincosamides	MY	6 (100)	1 (100)
	CD	6 (100)	1 (100)
Chloramphenicol	C	3 (50)	1 (100)
Quinolones	ENR	1 (17)	0 (0)
	CIP	6 (100)	1 (100)
	MAR	6 (100)	1 (100)

Antibiotic concentrations, reported in parentheses, are expressed in micrograms. *TS, Sulfa-trimethoprim (25); PG, Penicillin G (10); AP, Ampicillin (25); A, Amoxicillin (25); AMC, Amoxicillin \clavulanate (30); CDX, Cefadroxil (30); CL, Cefalexin (30); KZ, Cefazolin (30); CVN, Cefovecin (30); OT, Oxytetracycline (30); DXT, Doxycycline (30); S, Streptomycin (10); K, Kanamycin (30); GM, Gentamycin (10); AK, Amikacin (30); E, Erythromycin (15); TY, Tylosin (30); MY, Lincomycin (15); CD, Clindamycin (2); C, Chloramphenicol (30); ENR, Enrofloxacin (5); CIP, Ciprofloxacin (5); MAR, Marbofloxacin (5).

inhabit the ocular surface of many animal species without any appreciable symptom (Murphy *et al.* 1978; Foti *et al.* 2013).

From cloacal swabs, we observed mostly Gram-negative isolates, in particular, *E. coli*, *Proteus* spp. and *Klebsiella* spp. *E. coli* and *Proteus* spp. are considered normal components of the intestinal bacterial flora of captive raptors (Bangert *et al.* 1988; Lamberski *et al.* 2003). However, under stressful conditions, these bacteria may cause opportunistic infections involving different organs and tissues (Gerlach 1994). Of particular interest is the isolation in our samples of *Escherichia fergusonii* and *S. odorifera* (Bangert *et al.* 1988), previously reported in captive raptors and in healthy and sick chickens (Forgetta *et al.* 2012; Oh *et al.* 2012). *S. odorifera* was also isolated in diseased dogs (Lee *et al.* 2006; Yamada *et al.* 2013). Among our isolates other findings, such as *Citrobacter youngae*, *Providencia stuartii* and *Raoultella ornithinolytica* are reported within the gut flora of wild birds (Hernandez *et al.* 2003) and have been isolated from diseased animals (Waldhalm *et al.* 1969). The profile of antibiotic resistance of our isolates deserves special attention, because all our isolates were MDR. Even if these data are in agreement with previous studies on wild birds, including raptors (Tosi *et al.* 2014; Sousa *et al.* 2014), the extent of multi-resistant isolates detected in this study was unexpected. Moreover, this is the first report on MDR isolates in raptors in a zoological garden. Although it is difficult to explain how AMR isolates developed, also because these animals do not undergo antibiotic treatments, nevertheless we can still make some considerations. In general terms, the transmission of AMR may be due to the spread of whole AMR bacteria or to horizontal gene transfer, both at level of animal hosts and in terrestrial and aquatic habitats (Dröge *et al.* 1999; Marshall & Levy 2011). In our case, some elements of risk can be considered. The first element concerns the food given to animals. Rabbits, quails, mice and rats were bred in internal facilities and massive external contamination can be excluded. Chicken thighs and turkey wings were of high quality and fit for human consumption, meeting rigorous food safety and hygiene specifications. Chicks were purchased and no further microbiological or toxicological control was performed. A second element is related to the exchange of birds of prey between zoos or their shift from one enclosure to another, within the zoo, that occasionally occurs due to a renewal of an enclosure or changes in the collection plan. In these cases, cages are washed and disinfected as well as possible, but a complete disinfection is not easy to obtain because cages are furnished (i.e. perches of natural wood, natural bushes, stones, natural floor, etc.). Incoming raptors can only come from zoos that meet the requirements of the Directive 1999/22/CE – D. L.vo 73/2005. For these

Table 3. Antibiotic resistance of bacterial isolates from cloacal swabs

Antibiotics		Number (%) of resistant isolates					
		Escherichia coli (*N* = 14)	*Escherichia fergusonii* (*N* = 2)	*Proteus* (*N* = 6)	*Klebsiella* (*N* = 7)	*Staphylococcus aureus* (*N* = 4)	Coagulase-negative *Staphylococci* (*N* = 2)
Sulfonamides	TS*	11 (79)	0 (0)	2 (33)	7 (100)	2 (50)	1 (50)
Penicillins	PG	13 (93)	2 (100)	6 (100)	7 (100)	4 (100)	1 (50)
	AP	8 (57)	2 (100)	6 (100)	7 (100)	4 (100)	1 (50)
	A	8 (57)	0 (0)	5 (83)	7 (100)	2 (50)	0 (0)
	AMC	9 (64)	2 (100)	2 (33)	4 (57)	4 (100)	1 (50)
Cephalosporins	CDX	12 (86)	2 (100)	3 (50)	7 (100)	2 (50)	0 (0)
	CL	12 (86)	0 (0)	6 (100)	7 (100)	3 (75)	1 (50)
	KZ	7 (50)	2 (100)	6 (100)	6 (85)	4 (100)	2 (100)
	CVN	1 (7)	2 (100)	2 (33)	0 (0)	2 (50)	0 (0)
Tetracyclines	OT	9 (64)	0 (0)	6 (100)	7 (100)	2 (50)	1 (50)
	DXT	10 (71)	2 (100)	6 (100)	7 (100)	4 (100)	2 (100)
Aminoglycosides	S	8 (57)	2 (100)	6 (100)	NT†	4 (100)	2 (100)
	K	13 (93)	2 (100)	6 (100)	7 (100)	4 (100)	2 (100)
	GM	12 (86)	2 (100)	6 (100)	7 (100)	4 (100)	2 (100)
	AK	12 (86)	2 (100)	6 (100)	NT	4 (100)	2 (100)
Macrolides	E	14 (100)	2 (100)	6 (100)	7 (100)	4 (100)	2 (100)
	TY	14 (100)	2 (100)	6 (100)	7 (100)	4 (100)	2 (100)
Lincosamides	MY	14 (100)	2 (100)	6 (100)	7 (100)	4 (100)	2 (100)
	CD	13 (93)	2 (100)	6 (100)	7 (100)	4 (100)	1 (50)
Chloramphenicol	C	1 (7)	0 (0)	3 (50)	NT	3 (75)	0 (0)
Quinolones	ENR	9 (64)	0 (0)	6 (100)	6 (85)	2 (50)	0 (0)
	CIP	11 (79)	2 (100)	2 (33)	7 (100)	4 (100)	2 (100)
	MAR	6 (43)	0 (0)	2 (33)	6 (85)	3 (75)	1 (50)

Antibiotic concentrations, reported in parentheses, are expressed in micrograms. *TS, Sulfa-trimethoprim (25); PG, Penicillin G (10); AP, Ampicillin (25); A, Amoxicillin (25); AMC, Amoxicillin\clavulanate (30); CDX, Cefadroxil (30); CL, Cefalexin (30); KZ, Cefazolin (30); CVN, Cefovecin (30); OT, Oxytetracycline (30); DXT, Doxycycline (30); S, Streptomycin (10); K, Kanamycin (30); GM, Gentamycin (10); AK, Amikacin (30); E, Erythromycin (15); TY, Tylosin (30); MY, Lincomycin (15); CD, Clindamycin (2); C, Chloramphenicol (30); ENR, Enrofloxacin (5); CIP, Ciprofloxacin (5); MAR, Marbofloxacin (5).†NT, not tested.

animals, quarantine is not required. It is therefore not possible to exclude the risk of spread of AMR bacteria, especially if non-pathogenic, from the incoming animals. Regarding the role of the environment in the dissemination of antibiotic resistance, it has been reported that gene-exchange through conjugation, transformation or transduction also occur in nature (Dröge *et al.* 1999). Moreover, usage of antimicrobials in food animals represents a substantial proportion of the overall consumption of antimicrobials worldwide and antibiotics, as well as antibiotic-resistant bacteria, can be released into the environment from animal farms. The zoo in this study is located at the northern border of the Po river valley, between the neighbouring Adige river and Garda lake. However, the zoo is not in direct contact with these sources of water and animals are watered with tap water. There are many intensive cattle, pig and poultry farms in the Po valley. In the area where the zoo is located, however, there are mostly vineyards. In conclusion, all the considered factors cannot be quantified and do not allow to establish whether and how the presence of MDR bacteria detected in birds of prey can be attributable to the environment and to the small wild animals present in it. Anyhow, considering our results, we think that these birds could represent a risk for the transmission of MDR bacteria to other animal species. This should be taken into consideration when designing the layout of a multi-species exhibit or when planning the transfer of birds from one zoo to another.

Conclusions

1. Despite the low number of subjects tested, to the author's knowledge this is the first report regarding the presence of MDR strains within raptors housed in a zoological garden.
2. Since resistance genes can be transferred to other pathogenic bacteria, there is a potential hazard for the emergence of new multidrug-resistant pathogens, especially in a limited environment such as those of a zoo.
3. The transfer of animals between different zoos, or their possible release back to the wildlife, could lead to a widespread of MDR bacteria even in geographically different areas.
4. Monitoring of microbial flora of these animals could be useful to preserve the health of the endangered species housed in a zoo and improve *ex-situ* conservation programmes.

Acknowledgements

The authors thank Caterina Spiezio for critical reading of the manuscript.

Source of Funding

No external funding was required.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Contributions

The authors have no additional contributions to declare.

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