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Authors: Marrow, Judilee, Whittington, Julia K., Mitchell, Mark, Hoyer, Lois L., and Maddox, Carol

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PREVALENCE AND ANTIBIOTIC-RESISTANCE CHARACTERISTICS OF *ENTEROCOCCUS* SPP. ISOLATED FROM FREE-LIVING AND CAPTIVE RAPTORS IN CENTRAL ILLINOIS

Judilee Marrow,[1] Julia K. Whittington,[2,4] Mark Mitchell,[2] Lois L. Hoyer,[3] and Carol Maddox[3]

[1] College of Veterinary Medicine, University of Illinois, Urbana, Illinois 61802, USA
[2] Department of Veterinary Clinical Medicine, University of Illinois, Urbana, Illinois 61802, USA
[3] Department of Pathobiology, University of Illinois, Urbana, Illinois 61802, USA
[4] Corresponding author (email: jkwhitti@uiuc.edu)

ABSTRACT: Due to their predatory nature, raptor species may serve as important indicators of environmental contamination with antimicrobial-resistant bacteria. Raptors prey on small rodents and birds that have diverse habitat ranges, including urban and rural environments, and their intestinal microflora can reflect that of the animals on which they feed. *Enterococcus* spp. were selected as target organisms because they have been isolated from the avian gastrointestinal tract, can be conferred by prey items, and because they are capable of multiple resistance patterns. They are also a concerning source of human antimicrobial resistance. In this study fecal cultures were obtained from 15 May 2004 to 31 August 2004, from 21 free-living raptors and four captive raptors. *Enterococcus* was isolated from 21 (84%) of the 25 birds, and 54 isolates were chosen for further study based upon unique colony morphology. The most common isolate recovered was *Enterococcus faecalis* (95%, 95% confidence interval [CI]: 89–100). One bird in the study was determined to have *Enterococcus gallinarum*. Two distinct ribotypes of *E. faecalis* were identified, one with unique bands at 11 and 13 kb and the other with unique bands at 14 and 20 kb. Both ribotypes were found in free-living and captive birds. The *Enterococcus* isolates in this study demonstrated a variety of antimicrobial-resistance characteristics, including almost complete resistance to amikacin, first-generation cephalosporins, spectinomycin, and sulphadimethoxime. Isolates demonstrated variable resistance to chloramphenicol, gentamicin, enrofloxacin, erythromycin, and ticarcillin. No phenotypically vancomycin-resistant *E. faecalis* isolates were recovered from any of the raptors; three isolates had intermediate level susceptibility. A significantly higher number of isolates collected from captive birds demonstrated resistance to chloramphenicol than those obtained from free-living birds. This trend was not duplicated with any of the remaining 18 antimicrobial drugs tested. The results of this study suggest that raptors in central Illinois are coming into contact with antimicrobials, prey exposed to antimicrobials, or bacteria that are capable of transferring resistance genes. Further study is needed to determine the source of antimicrobial-resistant *Enterococcus* in free-living raptors but the limited data reflecting few differences between birds with and without antimicrobial exposure suggests that treatment and release of treated wild raptors is not contributing significantly to antimicrobial resistance in the environment.

Key words: Antimicrobial resistance, intestinal microflora, susceptibility, vancomycin.

INTRODUCTION

Human populations, animal populations, and the environment are all interconnected, and there is a blurring of the lines previously drawn that distinguished human diseases from animal diseases (Chomel, 1998). Emerging and re-emerging pathogens are on the rise, including the emergence of multiple-drug–resistant bacteria, and the role of potential reservoirs of pathogens is poorly understood (Woolhouse, 2002; Bengis et al., 2004). The development of antibiotic resistance among bacteria is a point of concern in both human and animal medicine. There are a number of factors that have been associated with increased development of antimicrobial resistance among bacteria as well as other emerging pathogens. Increased pathogenicity and antibiotic resistance patterns may result from genetic changes among bacterial strains (O'Brien, 2002). Changes in host populations, population health, and ecology also affect interactions with potential pathogens (Woolhouse, 2002). One of the leading causes of changes in host–pathogen ecology is globalization and technologic advances of the modern world. Frequent

empirical-based antibiotic therapy of humans, the addition of antibiotics as growth promotants in livestock feed, and antibiotic misuse have been implicated in the selective proliferation of resistant strains (Pantosti et al., 1999; Del Grosso et al., 2000; Mateu and Martin, 2001; O'Brien, 2002; Hershberger et al., 2005). The transmission of antimicrobial-resistant strains of pathogenic bacteria to humans is of increasing significance (Sørensen et al., 2001; Kuehn, 2007). In addition to direct colonization, public health costs include reduced efficacy of empirical antibiotic use, reduced choices for treatment, and possible coselection of virulence traits among pathogens (Mølbak, 2004).

Enterococcus spp. are known commensal organisms of the gastrointestinal tract and are also opportunistic pathogens associated with significant morbidity and mortality of humans and animals (Ellerbroek et al., 2004). Because of the prevalence of *Enterococcus* spp. in nosocomial infections, many have suggested that the hospital setting serves as the reservoir for antimicrobial-resistant strains (Ruiz-Garbajosa et al., 2006). Additional studies suggest environmental sources of the bacteria, including animals, can serve as important reservoirs for antimicrobial-resistant *Enterococcus* strains (Sørensen et al., 2001; Mallon et al., 2002). Studies have demonstrated a link between human sources of resistant strains of *Enterococcus* isolates and the environment, and have suggested that animals and their products are contaminated secondary to interaction with humans and the environment (Aarestrup et al., 1995; Iversen et al., 2004). These bacteria are of particular concern in human and animal medicine because some strains have constitutive antimicrobial resistance traits, and others carry inducible resistance traits (Eisner et al., 2005). Another concern is that these organisms can transfer resistance genes to other bacterial species including pathogens (Moubareck et al., 2003; Lester et al., 2006). Although opinions differ in defining the source of resistant *Enterococcus* spp., increasing development of antimicrobial resistance and transferable resistance genes are points of concern (O'Brien, 2002).

With urban expansion, the interface between wild animals and humans has increased dramatically. Some species, such as small rodents and flocking birds, can thrive in both rural and urban areas, and may serve as reservoirs for antimicrobial-resistant bacteria potentially transferring pathogens between these two environments (Sellin et al., 2000; Mallon et al., 2002; Walderström et al., 2005). In the past, raptors have been used as indicators for environmental contamination from pesticides and heavy metals; however, their predatory relationship with small wildlife species may also allow them to be used as indicators of antimicrobial resistance present in bacteria encountered in their environment (Smith et al., 2002; Jaspers et al., 2006). With the use of raptors as sentinels, wildlife hospitals and rehabilitation centers can screen animals to qualify and quantify the potential pathogen load carried by the animal, and can infer information about the environment from which the bird originated. In this way, trends in antibiotic susceptibility and developing resistance patterns of environmental bacteria may be monitored (Steele et al., 2005). In these centers, individual patients are often treated with prolonged antimicrobial therapy, which may contribute to the development of antimicrobial-resistant gastrointestinal flora and the establishment of nosocomial pathogens. Monitoring for antimicrobial resistance development in hospitalized or captive individuals will help elucidate whether wildlife rehabilitation is contributing to environmental contamination with antimicrobial-resistant strains of bacteria.

The goals of this study were to determine if enterococci can be cultured from the gastrointestinal flora of birds of prey

and with what frequency it was isolated. *Enterococcus* isolates recovered from the gastrointestinal tract of free-living raptors were characterized by genotype and antimicrobial phenotype, with the use of minimal inhibitory concentration (MIC) testing. These data were then compared to limited data available for captive raptors and those treated with antimicrobial therapy in a captive care setting.

MATERIALS AND METHODS

This study was conducted from 15 May 2004 to 31 August 2004 with free-living raptors and captive native resident raptors from the University of Illinois Wildlife Medical Clinic (WMC; Urbana, Illinois, USA). Free-living raptors in need of veterinary care were presented by concerned citizens or the Illinois Department of Natural Resources. Animals were presented with limited histories, but were excluded from this study if they had received treatment with antibiotics prior to presentation. All free-living raptors were found within 160 km of the WMC (40°6.4′N, 88°11.7′W). The habitat is comprised of small to medium residential communities surrounded by rural agricultural areas. The captive raptors, used in an education program and permanently housed at the WMC, were also evaluated in the study for the purposes of comparison. All captive raptors had been in captivity for greater than 1 yr and had not received antibiotics within 6 mo at the time of this study. The captive raptors are housed in the same ward as the free-living hospitalized patients, and all birds are housed individually in standard hospital stainless-steel cages. The sampling of raptors was performed in accordance with the regulations established by the University of Illinois Animal and Care and Use Committee (Protocol No. 04177).

Fecal samples were obtained for culture from the cloaca with the use of culture swabs with Stuart transport medium. Free-living raptors were sampled at the time of their admission. When antimicrobial therapy was implemented postadmission, subsequent samples were collected at 72–96 hr postinitiation of therapy and 7 days postcessation of therapy when possible. Not all treated raptors were sampled the maximum number of times, because of death or release of the patient. The captive birds were sampled at the same time intervals, but were not treated with antimicrobials. Specimens were originally plated on colistin-nalidixic acid (CNA) agar with 5% sheep blood (a gram-positive selective agar) or M-*Enterococcus* agar (an *Enterococcus*-selective agar). A minimum of four colonies, two from the M-agar and two from the CNA plates, were selected from each specimen for further characterization, whenever possible, to increase the chances of selecting unique isolates. Phenotypically, colonies were considered as probable *Enterococcus* if they were catalase-negative, demonstrated hemolysis, demonstrated growth in 6.25% sodium chloride broth, and were comprised of gram-positive cocci. All prepared plates and broth were purchased from Remel, Inc. (Lenexa, Kansas, USA).

Once isolates were identified as *Enterococcus* based on phenotypic tests, one–four colonies with unique morphology from each animal were chosen for further characterization. Antimicrobial susceptibility of the isolates was determined with the use of commercially prepared CMV2ECOF companion animal/equine microwell broth dilution panels (Sensititre®, TREK, Westlake, Ohio, USA) to determine the minimum inhibitory concentration (MIC). Vancomycin MIC values were determined using the E-test gradient strip method (AB BioDisk, Piscataway, New Jersey, USA). All susceptibility test methods were performed as recommended by the manufacturer and published standards (Clinical and Laboratory Standards Institute [CLSI], 2002). Interpretations and breakpoints for susceptible, intermediate, and resistance levels follow CLSI published data (CLSI, 2008).

All isolates were confirmed to be *Enterococcus* spp. by polymerase chain reaction (PCR) with the use of the primers listed in Table 1 (minor modifications of those published by Ke et al., 1999; Dutka-Malen et al., 1995; Angeletti et al., 2001). Bacterial DNA was purified with the use of the QiAmp® kit (Qiagen, Inc., Valencia, California, USA) protocol for bacterial cultures. Thermocycler parameters were as follows: strand separation at 94 C for 5 min, followed by 29 cycles of denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec, and extension at 72 C for 1 min and 72 C for 7 min. Tubes were held at 4 C until loaded into Ready 3% Agarose Mini Gels with ethidium bromide (Bio-Rad Laboratories, Hercules, California, USA). Gels were electrophoresed in 1× tris-borate EDTA buffer at 180 V for 90 min, then photographed with the use of a UV light box and Polaroid 667 film. Polymerase chain reaction was also used to test *Enterococcus* isolates for the presence of vancomycin-resistance genes. Polymerase chain reaction primers and reaction conditions for detection of *vanA*, *vanB*, *vanC-1*, and

TABLE 1. *Enterococcus* speciation PCR primers.

Primer	Sequence	Amplicon size (base pairs)
Enterococcus sp.		482
tuf F	5′-CTT AGT AGT TTC TGC TGC TGA TTT CAA CAC CTG TAA CGG-3′	
tuf R	5′-GGA ACA TTT CAA CAC CTG TAA CGG-3′	
Enterococcus faecium		658
E fcm1	5′-TTG AGG CAG ACC AGA TTC ACG-3′	
E fcm 2	5′-TAT GAC AGC GAC TCC GAT TCC-3′	
Enterococcus faecalis		941
E fcl 1	5′-ATC AAG TAC AGT TAB TCT TTA TTA G-3′	
E fcl 2	5′-ACG ATT CAA AGC TAA CTG AAT CAT T-3′	

vanC-2/3 were described by Patel et al. (1997). All four primer pairs were multiplexed in a single reaction that allowed detection of *vanA/vanB* as a fragment of unique size and the *vanC* products as another fragment. Results are reported in this manner. The genetic ribotype of each isolate was determined with the use of the *Eco*RI digest protocol for the DuPont Qualicon RiboPrinter® (DuPont, Wilmington, Delaware, USA). Isolates were ribotyped by the University of Illinois Veterinary Diagnostic Laboratory Microbiology section with the use of the RiboPrinter® instructions.

The binomial 95% confidence intervals (95% CI) were determined for each proportion. Fisher's exact test was used to compare the prevalence of *Enterococcus* spp. between the free-living raptors and captive raptors. The Mann-Whitney (MW) test was used to compare antibiotic resistance patterns for each antibiotic between age groups, free-living/captive status, and ribotype, and the Kruskal-Wallis test was used to compare antibiotic resistance patterns between the different species of birds presented. The chi-square test for independence was used to determine if there were differences in ribotype or phenotypic resistance to vancomycin by raptor species. The chi-square test for independence was also used to determine if the different types of media used were predictive of the ribotype isolated. The analysis was done with the use of SPSS 15.0 (SPSS, Inc., Chicago, Illinois, USA). A $P<0.05$ was used to determine statistical significance.

RESULTS

Twenty-five birds were enrolled in this study: 21 free-living raptors including seven Red-tailed Hawks (*Buteo jamaicensis*), eight American Kestrels (*Falco sparverius*), three Great Horned Owls (*Bubo virginianus*), one Eastern Screech Owl (*Otus asio*), one Osprey (*Pandion haliaetus*), and one Barred Owl (*Strix varia*), in addition to four raptors maintained in captivity, including one Red-tailed Hawk, one American Kestrel, one Great Horned Owl, and one Eastern Screech Owl. The majority (80%, 20/25) of the birds were adults. The remaining birds were either juvenile or their age was indeterminate. Bird gender was not determined. During the course of the study, three of the wild raptors (two Red-tailed Hawks and one Great Horned Owl) were treated with antimicrobials as part of their medical therapy and rehabilitation. Cultures of the gastrointestinal tract of these birds during and subsequent to treatment were evaluated to determine the effects of antibiotic treatment, and prolonged exposure to the clinic environment, on the prevalence and sensitivity characteristics of *Enterococcus* isolates. The small number of isolates obtained from birds undergoing or having completed antimicrobial therapy, in addition to the isolates collected from the captive birds that had prolonged exposure to the clinic environment, were compared to those isolated from the antimicrobial naïve birds.

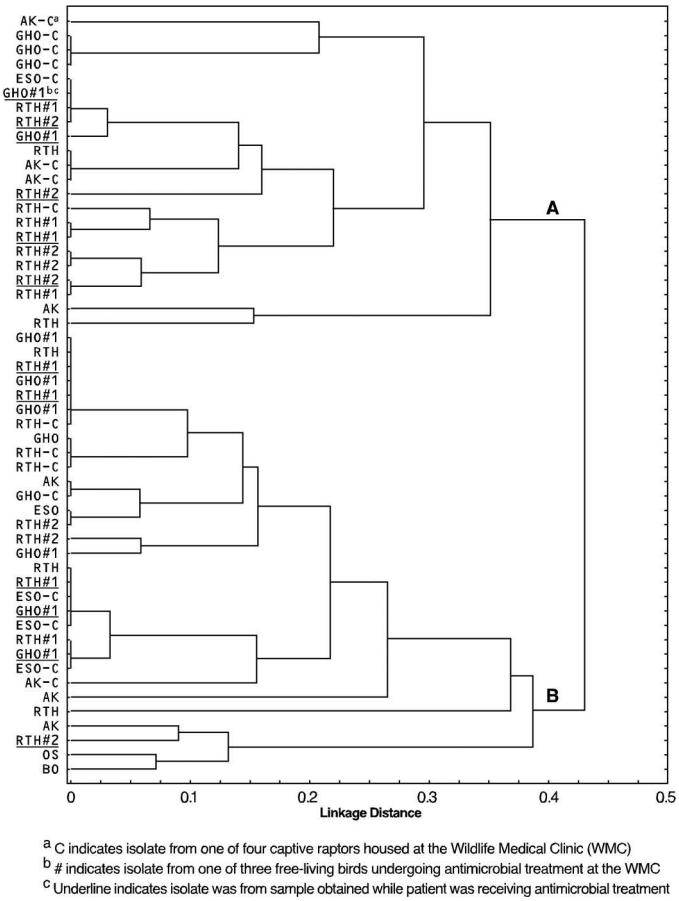
Enterococcus was cultured from 17/21 (81%, 95% CI: 64–98) free-living raptors and four of four (100%) captive raptors.

There was no significant difference in the prevalence of *Enterococcus* between the free-living and captive raptors ($P=1.0$). The overall prevalence between the groups was pooled (84%, 95% CI: 70–98). *Enterococcus* was not cultured from four of the eight free-living American Kestrels. From the 21 birds found to be *Enterococcus* positive, the predominant species recovered was *Enterococcus faecalis*, comprising 53/56 (95%, 95% CI: 88.6–100) of the isolates. The 56 isolates included 28 isolates collected from free-living raptors with no known antimicrobial exposure upon their admission to the WMC, 16 isolates collected from the captive residents of the WMC, and 12 isolates collected from three birds hospitalized and undergoing antimicrobial therapy. One of the raptors that was sampled upon admission, Great-Horned Owl MR No. 04-402, demonstrated three (5.3%, 95% CI: 0–11) isolates confirmed by ribotyping to be *Enterococcus gallinarum*. All three isolates were of identical ribotype and susceptibility profile, so only one of these isolates was included in the antibiotic susceptibility analysis. Fifty-four isolates (53 *E. faecalis*, one *E. gallinarum*) were chosen for further characterization. The *E. gallinarum* isolates were excluded from the hierarchic cluster analysis (HCA) of the Riboprint® data, as this resulted in an additional clade with no other members, reducing the ability to discriminate between members of Clades A and B (Fig. 1).

Minimum inhibitory concentration values for 19 drugs were determined for each of the 54 *Enterococcus* isolates; MIC values were compiled to determine significant differences from breakpoint values (Table 2). No statistical difference could be determined between isolates from birds with or without antibiotic exposure; thus samples were combined for the purpose of this study. Isolates demonstrated innate resistance to first-generation cephalosporins (>98% resistant), third-generation cephalosporins (96% resistant to ceftiofur), spectinomycin (98% resistant), amikacin (93% resistant), and sulphadimethoxime (100% resistant). Isolates demonstrated variable antimicrobial resistance patterns for enrofloxacin (40% resistant), erythromycin (50% resistant), chloramphenicol (39% resistant), tetracycline (33% resistant), and ticarcillin (37% resistant). Isolates were susceptible to penicillins (0% resistant) and potentiated penicillins (0% resistant). None of the 53 *E. faecalis* isolates or the single *E. gallinarum* isolate was resistant to vancomycin based upon the CLSI (2008) breakpoints for enterococci. Isolates with MIC values less than or equal to 4 μg/ml were sensitive, those with MICs of 5–31 μg/ml were considered intermediate, and isolates with MICs greater than or equal to 32 μg/ml were considered resistant. The average vancomycin MIC value for these 54 isolates was 2.94 μg/ml, with a range of 1.5–12.0 μg/ml. Two intermediately vancomycin-susceptible isolates (MIC=5 μg/ml) were recovered, one from an untreated bird, the other from a resident. Another intermediately vacomycin-susceptible isolate (MIC=12 μg/ml) was the *E. gallinarum* isolate recovered from an untreated bird. The MIC90 value, the concentration at which ≥90% of the isolates are susceptible, to vacomycin for these 54 isolates is 8 μg/ml.

There was no difference in antimicrobial-resistance frequencies between the different age groups or species of birds. There was a significant difference (MW=283, $P=0.003$) in the antibiotic-resistance frequency for chloramphenicol between free-living and captive animals, with a larger proportion of the organisms isolated from captive birds (93%) being resistant to the drug compared with the free-living birds (58%). There were no other differences in the prevalence of antimicrobial resistance between species for any of the other drugs tested.

Thirty-nine of the isolates were positive for either *vanA/vanB* or *vanC* PCR products (72%, 95% CI: 60–84). Although amplicons were of the appropriate size,



[a] C indicates isolate from one of four captive raptors housed at the Wildlife Medical Clinic (WMC)
[b] # indicates isolate from one of three free-living birds undergoing antimicrobial treatment at the WMC
[c] Underline indicates isolate was from sample obtained while patient was receiving antimicrobial treatment

FIGURE 1. Hierarchic cluster analysis of *Enterococcus faecalis* ribotypes: 53 isolates of *E. faecalis* distributed between ribogroups A and B. Ribogroup B is the dominant group and there was no significant difference between the distribution between groups of isolates from free-living raptors without antimicrobial exposure and those housed in a clinic setting or with known antimicrobial treatment. Key: American Kestrel (AK), Great Horned Owl (GHO), Eastern Screech Owl (ESO), Red-tailed Hawk (RTH), Osprey (OS), Barred Owl (BO). Relative linkage relationships shown.

90% resulted in *van*A/*van*B amplicons, 36% tested positive for *vanC* genes (data not shown). There was a poor correlation between the vancomycin-resistance gene PCR results and the vancomycin MIC values determined by E-test.

DuPont® riboprinting detected two clades of isolates for the *E. faecalis* (Fig. 1). These were distinguished by unique bands at 11 and 13 kb (Group A) and 14 and 20 kb (Group B). At least six additional bands were conserved. Group B was the dominant ribotype identified, comprising 59% of *E. faecalis* isolates. There was no significant difference ($\chi^2=1.9$, $P=0.6$) in the likelihood of recovering the different ribotypes from the different media. Free-living birds were more likely ($\chi^2=8.3$, $P=0.01$) to have enterococci organisms belonging to Group B (56%, 95% CI: 42–70) than Group A (12%, 95% CI: 1–25). There was no difference in the clade group for captive birds (Group A: 50%, 95% CI: 25.5–74.5; Group B: 44%, 95% CI: 19–70) or for free-living birds undergoing antimicrobial treatment (Group A: 33%, 95% CI: 27–60; Group B: 33%, 95% CI: 27–60). Ribotype Group B was significantly ($\chi^2=6.5$, $P=0.03$) more likely to be resistant to

TABLE 2. Susceptibility prevalence (%) for *Enterococcus* isolates as determined by Sensititre CMV2ECOF (Sensititre, TREK, Westlake, Ohio, USA) or E-test (AB BioDisk, Piscataway, New Jersey, USA); $N=54$.

Antimicrobial concentration per microliter	0.25	0.5	1	2	4	8	16	32	64	256	# res[a]	% resistant
Amikacin						2[b]	**7**[c]				50	93
Amoxicillin/clavulate					100						0	0
Ampicillin	4	15	78	96	100						0	0
Cefazolin						**2**					53	98
Cefoxitin				2[d]							53	98
Ceftiofur		4									53	96
Cephalothin						**2**					53	98
Chloramphenicol					2	**61**					21	39
Enrofloxacin		24	28	59							22	41
Erythromycin	4	20	20	50							27	50
Gentamicin				2	30						38	70
Imipenem			57	96	98						1	2
Penicillin			2	39	94	100					0	0
Spectinomycin								2			53	98
Sulphadimethoxime											54	100
Tetracycline			65	67							18	33
Ticarcillin						2	**4**	**7**	**63**[e]		20	37
Ticarcillin/clavulate						2		**4**	**67**		18	33
Vancomycin[f]				54	41	**4**	**2**				0	0

[a] Number of resistant isolates.

[b] Lightface, % of organisms below Clinical and Laboratory Standards Institute (CLSI) guidelines for breakpoint minimum inhibitory concentration (MIC) sensitivity.

[c] Boldface, % of organisms above CLSI guidelines for breakpoint MIC sensitivity.

[d] When no boldface percentage is present, the MIC of the organisms exceeded the highest concentration of that drug on the panel, thus there is no defined susceptible concentration and the MIC was beyond breakpoint.

[e] Multiple boldface values indicate intermediate susceptibilities.

[f] Vancomycin intermediate breakpoint from 5 μg/ml to16 μg/ml, resistant at >16 μg/ml.

erythromycin (81%, 95% CI: 66–96) than Group A (50%, 95% CI: 27–73). There were no significant differences in the antimicrobial resistance frequencies for either ribotype for the other antibiotics.

DISCUSSION

In this study, *Enterococcus* specimens were readily cultured from the gastrointestinal tracts of free-living and captive raptors. These bacteria, with both antimicrobial-resistant and susceptible patterns, have previously been isolated from cloacal swab specimens or feces of free-living avian and mammalian species (Livermore et al., 2001). Isolation of enterococci from free-living raptors has been reported. *Enterococcus* isolates from captive Houbara Bustards (*Chlamydotic undulate*) were detected in over 80% of the samples collected (Silvanose et al., 1998), and *E. faecalis* was found to be a commensal organism of the gastrointestinal tract of Kori Bustard (*Ardeotis kori*) chicks up to 70 days of age (Naldo et al., 1998). *Enterococcus faecalis* and *Enterococcus faecium* were recorded with prevalences of >40% in Red Kites (*Milvus milvus*) and a prevalence ranging from 0% to 63.6% in Egyptian Vultures (*Neophron percnopterus*) free living in several areas of Spain (Blanco et al., 2006, 2007). Prior to 1986, enterococci were included in the *Streptococcus* genus, but were later reclassified as a distinct genus to include those group D streptococci that demonstrated variable hemolytic properties and that grew in conditions of high pH, high salt concentrations, and a wide temperature range

(10–45 C; Facklam, 2002). One survey of the aerobic gastrointestinal flora of raptors reflects this earlier nomenclature by reporting a frequency of isolation ranging from 43% to 73% for *Streptococcus faecalis* and 7–53% for *Streptococcus faecium* in a variety of free-living raptor species (Bangert et al., 1988). A survey of cloacal bacterial flora in free-living and captive Red-tailed Hawks and Cooper's Hawks (*Accipiter cooperii*) reported higher frequencies of isolation of *Streptococcus* spp., 100% in captive birds and 50–70% in free-living birds, but did not pursue additional testing to identify enterococci positively (Lamberski et al., 2003). The frequency of isolation of *Enterococcus* in our study, predominantly *E. faecalis*, is much higher than previously reported, which may indicate an increase in prevalence of *Enterococcus* in raptor populations of central Illinois or may be attributed to successful use of differential and selective media. *Enterococcus faecalis* was the predominant species recovered, although *E. gallinarum* was recovered from one Great Horned Owl. *Enterococcus gallinarum* is an isolate most often associated with birds, whereas *E. faecalis* is primarily associated with mammals, such as small rodents (Mallon et al., 2002). Another variation in bacterial recovery was noted when American Kestrels were cultured; *E. faecalis* was only recovered from four of eight individuals included in this study, and all four individuals were wild animals. Among raptors included in this study, American Kestrels are unique from the other species sampled because their diet is more diverse and includes prey items such as small rodents, birds, and insects. Variation in gastrointestinal *Enterococcus* recovery from wild and captive raptors may be influenced by prey sources or differences in the microenvironment of the gastrointestinal tract of individual birds. Several studies support the belief that gastrointestinal flora reflect the diet, feeding habits, geography, and microenvironment of the prey. Two studies found that scavengers, such as raptors feeding on carrion, have significantly different gastrointestinal microflora than those of the same species feeding on live prey. The cloacal microflora isolated from birds in these studies was comprised of commensal intestinal bacteria, bacteria commonly isolated from their respective prey, and bacteria associated with the geography and ecology of the environment. Additionally, antibiotic resistance patterns were detected more frequently in bacteria isolated from raptors feeding on discarded livestock (Blanco et al., 2006, 2007). Fecal cultures from captive raptors fed diets containing poultry carcasses demonstrate several bacteria associated with poultry products and meat that were not found or were found less frequently in raptors fed a rodent diet (Bangert et al., 1988). Additional studies indicate that omnivorous diets may predispose animals to increased prevalence of certain bacteria, including *Enterococcus* spp. (Brittingham et al., 1988; Livermore, 2001) and that geographic location can have a bearing on the inhabitants' gastrointestinal microflora (Soucek and Mushin, 1970). Further studies are needed to explain how prey selection and microenvironment of the gastrointestinal tract contributes to the natural flora of raptors.

Genetic variation within a species of bacteria (ribotypes) has been used to differentiate between nosocomial bacterial populations and naturally occurring isolates (Woodford et al., 1993). In this study, no significant difference in antibiotic resistance characteristics could be determined between the two ribotype clades. Ribotypes were also evenly distributed between birds without previous antimicrobial exposure and those either treated with antibiotics or housed in the Wildlife Medical Clinic. This result indicates that neither of the clades represents an obvious nosocomial hospital strain. The presence of the two ribotype groups identified in this study may be an indication of natural diversity of gastrointestinal flora in indi-

vidual raptors. Although gastrointestinal microenvironments of individual raptors may be more likely to harbor one ribotype over another, this was not demonstrated in this study, as frequently both ribotypes were recovered from the gastrointestinal tract of the same bird.

In this study, *Enterococcus* isolates demonstrated a variety of antimicrobial resistance characteristics, including almost complete resistance to aminoglycosides, cephalosporins, spectinomycin, and sulfa antimicrobials. Variable resistance characteristics were noted when isolates were tested for resistance to chloramphenicol, enrofloxacin, erythromycin, and tetracycline. Previous investigations have identified specific genes that confer innate resistance against aminoglycoside, cephalosporin, and spectinomycin antimicrobials (Clark et al., 1999; Jeljaszewics et al., 2000; Alam et al., 2005). Although a specific genetic mechanism has not been identified, Chenoweth et al. (1990) report that the in vitro susceptibility to trimethoprim sulfadimethoxine is a poor indicator of in vivo performance against *E. faecalis* JH2-2 carrying plasmid pAD1. In addition to innate resistance, *Enterococcus* spp. have demonstrated inducible resistance to antimicrobials such as chloramphenicol, enrofloxacin, erythromycin, and tetracycline (Huys et al., 2004; Leener et al., 2005). Many of these resistance genes are located on transferable sections of DNA (plasmids), which can be exchanged between different *Enterococcus* isolates (Huys et al., 2004). Although determining specific resistance genes for antimicrobial agents was outside the scope of this study, the phenotypic resistance characteristics seen in this study closely parallel previously reported genetic characteristics of *Enterococcus*. Our isolates also possess the same resistance patterns seen previously from veterinary and environmental sources (Ellerbroek et al., 2004; Middleton and Ambrose, 2005; Macovei and Zurek, 2006). No significant correlation between resistance patterns and the antimicrobial exposure group could be determined in this study, except for chloramphenicol. The significant finding of increased resistance among birds maintained in a captive environment may suggest previous exposure to chloramphenicol; however, this drug is not routinely used in this clinical setting. The chloramphenicol resistance gene may be genetically linked to other antimicrobial resistance genes for which there has been antimicrobial selection pressure. The multiple antimicrobial-resistant *Enterococcus* isolates recovered in this study may be due to environmental exposure to antimicrobial agents through prey sources, exchange of resistance genes in the environment, or exposure to medical therapy in rehabilitation centers. The fact that there are few differences between groups of birds suggests that treatment and release of treated wild raptors is not contributing significantly to antimicrobial resistance in the environment, but definitive conclusions cannot be drawn without further testing.

Vancomycin resistance is a significant point of concern when discussing antimicrobial resistance in *Enterococcus* spp. Specific genes that confer inducible, high-level vancomycin resistance (*vanA* and *vanB*) have been identified, and other genes that confer low-level innate resistance have been identified (*vanC-1/vanC-2*; Donabedian et al., 2006). Based upon guidelines for vancomycin-susceptibility breakpoints for enterococci (CLSI, 2008), a sensitive interpretation is applied for isolates with MICs$\leq$4.0 μg/ml. This interpretation leads to a smaller proportion of isolates that are not deemed sensitive (three of 54, 6%) with intermediate level susceptibility. Many more enterococci in our study carried vancomycin-resistance genes A/B or C (data not shown) than expressed a resistant phenotype. Amplicon sequencing, newer BLAST comparisons to GenBank, and further RT-PCR studies might help determine if failure to express resistance genes may account for the discrepancies between vancomycin genotype and phenotype or if

the selection of breakpoints underestimates the potential for vancomycin resistance.

It has been shown that free-living wildlife, especially waterfowl, may serve as reservoirs, and potentially sentinels, of zoonotic pathogens, most notably *Salmonella*, *Campylobacter*, and *Escherichia coli* (White and Forrester, 1979; Fallacara et al., 2001; Smith et al., 2002; Dobbin et al., 2005; Middleton and Ambrose, 2005). Free-living raptors also are important sentinels for potential pathogens found in their environment (Winsor et al., 1981; Steele et al., 2005). *Enterococcus faecalis* is responsible for the majority of human enterococcal infections, and was the predominant species recovered from raptors in our study. The *Enterococcus* isolates retrieved from raptors in this study demonstrated a variety of antimicrobial resistance patterns, including intermediate vancomycin susceptibility. Further identification of the variety of resistance genes and resistance mechanisms would be needed to completely characterize these isolates. Although outside the scope of this study, the high level of antimicrobial resistance in the nontreated group is of concern. The results of this study suggest that raptors in central Illinois are coming into contact with antimicrobials, prey exposed to antimicrobials, or bacteria that are capable of transferring resistance genes. Further exploration of the expression of vancomycin genes of *Enterococcus* strains is needed to determine if vancomycin resistance is innate and if it is being expressed by the gastrointestinal flora of wild and captive raptors. *Enterococcus faecalis* can cause acute septicemia or chronic subacute infections in birds (Naldo et al., 1998) but the impact that these bacteria have on raptors, and the impact that these birds have on the environment and public health warrants further study.

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LITERATURE CITED

Aarestrup, F. M. 1995. Occurrence of glycopeptide resistance among *Enterococcus faecium* isolates from conventional and ecological poultry farms. Microbial Drug Resistance 1: 255–257.

Alam, M. M., N. Kobayashi, I. Masaho, A. Sumli, K. Kobayashi, N. Uehara, and N. Watanabe. 2005. Detection of a novel aph (2′) Allele (*aph*[2″]-*Ie*) conferring high-level gentamicin resistance and a spectinomycin resistance gene *ant*(9)-*Ia* (*aad9*) in clinical isolates of enterococci. Microbial Drug Resistance 11: 239–247.

Angeletti, S., G. Lorino, G. Gheradi, F. Battistoni, M. De Cesaris, and G. Dicuonzo. 2001. Routine molecular identification of enterococci by gene specific PCR and 16S ribosomal DNA sequencing. Journal of Clinical Microbiology 39: 794–797.

Bangert, R. L., A. C. S. Ward, E. H. Stauber, B. R. Cho, and P. R. Widders. 1988. A survey of the aerobic bacteria in the feces of captive raptors. Avian Diseases 32: 53–62.

Bengis, R. G., F. A. Leighton, J. R. Fischer, M. Artois, T. Mörner, and C. M. Tate. 2004. The role of wildlife in emerging and re-emerging zoonoses. Revue Scientifique et Technique 23: 497–511.

Blanco, G., J. A. Lemus, and J. Grande. 2006. Faecal bacteria associated with different diets of wintering Red Kites: Influence of livestock carcass dumps in microflora alteration and pathogen acquisition. Journal of Applied Ecology 43: 990–998.

———, ———, ———, L. Gangoso, J. M. Grande, J. A. Donázar, B. Arroyo, O. Frías, and F. Hiraldo. 2007. Geographical variation in cloacal microflora and bacterial antibiotic resistance in a threatened avian scavenger in relation to diet and livestock farming practices. Environmental Microbiology 9: 1738–1749.

Brittingham, M. C., S. A. Temple, and R. M. Duncan. 1988. A survey of the prevalence of selected bacteria in wild birds. Journal of Wildlife Diseases 24: 299–307.

Chenoweth, C. E., K. A. Robinson, and D. R. Schaberg. 1990. Efficacy of ampicillin versus trimethoprim-sulfamethoxazole in a mouse model of lethal enterococcal peritonitis. Antimicrobial Agents and Chemotherapy 34: 1800–1802.

Chomel, B. B. 1998. New emerging zoonoses: A challenge and an opportunity for the veterinary profession. Comparative Immunology, Microbiology and Infectious Diseases 21: 1–14.

Clark, N. C., O. Olsvik, J. M. Swenson, C. A. Spiegel, and F. C. Tenover. 1999. Detection of a streptomycin/spectinomycin adenylytransfer-

ase gene (*aadA*) in *Enterococcus faecalis*. Antimicrobial Agents and Chemotherapy 43: 157–160.

Clinical and Laboratory Standards Institute (formerly NCCLS). 2002. M31-A2. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard. Second Edition 22, No. 6. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

———. 2008. M31-A3. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard. Third Edition 28, No. 8. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

Del Grosso, M., A. Caprioli, P. Chinzari, M. C. Fontana, G. Pezzotti, A. Manfrin, E. D. Giannatale, E. Goffredo, and A. Pantosi. 2000. Detection and characterization of vancomycin-resistant enterococci in farm animals and raw meat products in Italy. Microbial Drug Resistance 6: 313–318.

Dobbin, G., H. Hariharan, P. Y. Daoust, S. Hariharan, S. Heaney, M. Coles, L. Price, and C. A. Muckle. 2005. Bacterial flora of free-living Double-crested Cormorant (*Phalacrocorax auritus*) chicks on Prince Edward Island, Canada, with reference to enteric bacteria and antibiotic resistance. Comparative Immunology, Microbiology and Infectious Diseases 28: 71–82.

Donabedian, S. M., M. B. Perri, D. Vager, E. Hershberger, P. Malani, S. Simjee, J. Chow, E. N. Vergis, R. R. Muder, K. Gay, F. J. Angulo, P. Bartlett, and M. J. Zervos. 2006. Quinupristin-dalfopristin resistance in *Enterococcus faecium* isolates from humans, farm animals, and grocery store meat in the United States. Journal of Clinical Microbiology 44: 3361–3365.

Dutka-Malen, S., S. Evers, and P. Courvalin. 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. Journal of Clinical Microbiology 33: 1434.

Eisner, A., G. Feierl, G. Goriewicz, F. Dieber, H. Kessler, E. Marth, and J. Kofer. 2005. High prevalence of VanA-type vancomycin-resistance enterococci in Austrian poultry. Applied and Environmental Microbiology 71: 6407–6409.

Ellerbroek, L., K. N. Mac, J. Peters, and L. Hultquist. 2004. Hazard potential from antibiotic-resistant commensals like enterococci. Journal of Veterinary Medicine Series B 51: 393–399.

Facklam, R. 2002. What happened to the streptococci: Overview of taxonomic and nomenclature changes. Clinical Microbiology Reviews 15: 613–630.

Fallacara, D. M., C. M. Monahan, T. Y. Morishita, and R. F. Wack. 2001. Fecal shedding and antimicrobial susceptibility of selected bacterial pathogens and a survey of intestinal parasites in free-living waterfowl. Avian Diseases 45: 128–135.

Hershberger, E., S. F. Oprea, S. M. Donabedian, M. Perri, P. Bozigar, P. Bartlett, and M. J. Zervos. 2005. Epidemiology of antimicrobial resistance in enterococci of animal origin. Journal of Antimicrobial Chemotherapy 55: 127–130.

Huys, G., K. Haene, J. M. Collard, and J. Swings. 2004. Prevalence and molecular characterization of tetracycline resistance in *Enterococcus* isolates from food. Applied and Environmental Microbiology 70: 1555–1562.

Iversen, A., L. Kuhn, M. Rahman, A. Franklin, L. Burman, B. Olsson-Liljquist, E. Torell, and R. Mollby. 2004. Evidence for transmission between humans and the environment of a nosocomial strain of *Enterococcus faecium*. Environmental Microbiology 6: 55–59.

Jaspers, V., S. Voorspoles, A. Covaci, and M. Eens. 2006. Can predatory bird feathers be used as a non-destructive biomonitoring tool of organic pollutants? Biology Letters 2: 283–285.

Jeljaszewics, J., G. Mlynarczyk, and A. Mlynarczyk. 2000. Antibiotic resistance in Gram-positive cocci. International Journal of Antimicrobial Agents 16: 473–478.

Ke, D. F., J. Picard, F. Martineau, C. Menard, P. H. Roy, M. Ouellette, and M. G. Bergeron. 1999. Development of PCR assay for rapid detection of enterococci. Journal of Clinical Microbiology 37: 3497–3503.

Kuehn, B. M. 2007. Antibiotic-resistant "superbugs" may be transmitted from animals to humans. Journal of the American Medical Association 298: 2125–2126.

Lamberski, N., A. C. Hull, A. M. Fish, K. Beckmen, and T. Y. Morishita. 2003. A survey of the choanal and cloacal aerobic bacterial flora in free-living and captive Red-tailed Hawks (*Buteo jamaicensis*) and Cooper's Hawks (*Accipiter cooperii*). Journal of Avian Medicine and Surgery 17: 131–135.

Leener, E., A. Decostere, E. De Graef, H. Moyaert, and F. Haesebrouck. 2005. Presence and mechanism of antimicrobial resistance among enterococci from cats and dogs. Microbial Drug Resistance 11: 395–403.

Lester, C., N. Frimodt-Møller, T. Sørensen, D. Monnet, and A. Hammerum. 2006. In vivo transfer of the *vanA* resistance gene from and *Enterococcus faecium* isolate of animals origin to an *E. faecium* isolate of human origin in the intestines of human volunteers. Antimicrobial Agents and Chemotherapy 50: 596–599.

Livermore, D. M., M. Warner, L. M. C. Hall, V. I. Enne, S. J. Projan, P. M. Dunman, S. L. Wooster, and G. Harrison. 2001. Antibiotic resistance in bacteria from magpies (*Pica pica*) and rabbits (*Oryctolagus cuniculus*) from west Wales. Environmental Microbiology 3: 658–661.

MACOVEI, L., AND L. ZUREK. 2006. Ecology of antibiotic resistance genes: Characterization of enterococci from houseflies collected in food settings. Applied and Environmental Microbiology 72: 4028–4035.

MALLON, D., J. CORKILL, S. HAZEL, J. S. WILSON, N. FRENCH, M. BENNETT, AND C. A. HART. 2002. Excretion of vancomycin resistant enterococci by wild mammals. Emerging Infectious Diseases 8: 636–638.

MATEU, E., AND M. MARTIN. 2001. Why is antimicrobial resistance a veterinary problem as well? Journal of Veterinary Medicine Series B 48: 569–581.

MIDDLETON, J. H., AND A. AMBROSE. 2005. Enumeration and antibiotic resistance patterns of fecal indicator organisms isolated from migratory Canada Geese (*Branta candadensis*). Journal of Wildlife Diseases 41: 334–341.

MØLBAK, K. 2004. Spread of resistant bacteria and resistance genes from animals to humans—the public health consequences. Journal of Veterinary Medicine Series B 51: 364–369.

MOUBARECK, C., N. BOURGEOIS, P. COURVALIN, AND F. DOUCET-POPULAIRE. 2003. Multiple antibiotic resistance gene transfer from animal to human enterococci in the digestive tract of gnotobiotic mice. Antimicrobial Agents and Chemotherapy 47: 2993–2996.

NALDO, J. L., C. D. SILVANOSE, J. H. SAMOUR, AND T. A. BAILEY. 1998. Developmental intestinal aerobic microflora in the Kori Bustard (*Ardeotis kori*). Avian Pathology 27: 359–365.

O'BRIEN, T. F. 2002. Emergence, spread, and environmental effect of antimicrobial resistance: How use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. Clinical Infectious Diseases 34(3 Suppl): S78–S84.

PANTOSTI, A., M. DEL GROSSO, S. TAGLIABUE, A. MACRI, AND A. CAPRIOLI. 1999. Decrease of vancomycin-resistant enterococci in poultry meat after avoparcin ban. The Lancet 354: 741–742.

PATEL, R., J. R. UHL, P. KOHNER, M. K. HOPKINS, AND F. R. COCKERILL III. 1997. Multiplex PCR detection of *vanA*, *vanB*, *vanC-1*, and *vanC-2/3* genes in enterococci. Journal of Clinical Microbiology 35: 703–707.

RUIZ-GARBAJOSA, P., M. J. BONTEN, D. A. ROBINSON, J. TOP, S. R. NALLAPAREDDY, C. TORRES, T. COQUE, R. CANTON, F. BAQUERO, B. E. MURRAY, R. DEL CAMPO, AND R. J. WILLEMS. 2006. Multilocus sequence typing for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. Journal of Clinical Microbiology 44: 2220–2228.

SELLIN, M., H. PALMGREN, T. BROMAN, S. BERGSTROM, AND B. OLSEN. 2000. Involving ornithologists in the surveillance of vancomycin-resistant enterococci. Emerging Infectious Diseases 6: 87–88.

SILVANOSE, C. D., T. A. BAILEY, J. H. SAMOUR, AND J. L. NALDO. 1998. Intestinal protozoa and associated bacteria in captive Houbara Bustards (*Chlamydotis undulatata*) in the United Arab Emirates. Avian Pathology 28: 94–97.

SMITH, W. A., J. A. K. MAZET, AND D. C. HIRSH. 2002. *Salmonella* in California wildlife species: Prevalence in rehabilitation centers and characterization of isolates. Journal of Zoo and Wildlife Medicine 33: 228–235.

SØRENSEN, T. L., M. BLOM, D. L. MONNET, N. FRIMODT-MØLLER, R. L. POULSEN, AND F. ESPERSEN. 2001. Transient intestinal carriage after ingestion of antibiotic-resistant *Enterococcus faecium* from chicken and pork. New England Journal of Medicine 345: 1161–1166.

SOUCEK, Z., AND R. MUSHIN. 1970. Gastrointestinal bacteria of certain Antarctic birds and mammals. Applied Microbiology 20: 561–566.

STEELE, C. M., R. N. BROWN, AND R. G. BOTZLER. 2005. Prevalences of zoonotic bacteria among seabirds in rehabilitation centers along the pacific coast of California and Washington, USA. Journal of Wildlife Disease 41: 735–744.

WALDERSTRÖM, J., D. MEVIUS, K. VELDMAN, T. BROMAN, D. HASSELQUIST, AND B. OLSEN. 2005. Antimicrobial resistance profiles of *Campylobacter jejuni* isolates from wild birds in Sweden. Applied and Environmental Microbiology 71: 2438–2441.

WHITE, F. H., AND D. J. FORRESTER. 1979. Antimicrobial resistant *Salmonella* spp. isolated from Double-crested Cormorants (*Phalacrocorax auritus*) and Common Loons (*Gavia immer*) in Florida. Journal of Wildlife Diseases 15: 235–237.

WINSOR, D. K., A. P. BLOEBAUM, AND J. J. MATHEWSON. 1981. Gram-negative, aerobic, enteric pathogens among intestinal microflora of wild turkey vultures (*Cathartes aura*) in west central Texas. Applied and Environmental Microbiology 42: 1123–1124.

WOODFORD, N., D. MORRISON, A. JOHNSON, V. BRIANT, R. GEORGE, AND B. COOKSON. 1993. Application of DNA probes for rRNA and *vanA* genes to vancomycin-resistant enterococci. Journal of Clinical Microbiology 31: 653–658.

WOOLHOUSE, M. E. J. 2002. Population biology of emerging and re-emerging pathogens. Trends in Microbiology 10: S3–S7.

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