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Enterococcus Species Isolated from Northern Bobwhite (*Colinus virginianus*) Chicks: Species Distribution and Antibiotic Resistance

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Abstract: Intestinal fragments from newly hatched to seven-day-old Northern Bobwhite chicks (n = 39) were processed to isolate organisms belonging to the genus *Enterococcus*. Identification of the eighty-nine enterococci revealed forty-two *E. saccharolyticus*, twenty *E. malodoratus*, eight *E. pseudoavium*, seven *E. raffinosus*, six *E. faecalis*, two *E. mundtii*, two *E. avium*, one *E. cecorum* and one *E. durans*. Each isolate was examined for its resistance to ampicillin, ciprofloxacin, clindamycin, erythromycin, nitrofurantoin, tetracycline, and vancomycin by the disk diffusion assay. Our results indicate all isolates were resistant to at least two of the antibiotics examined, sixty-nine were resistant to three different antibiotics, while sixteen isolates were resistant to four of the seven antibiotics examined. Most significantly, eighteen enterococcal isolates, five of which were *E. faecalis*, were shown to be vancomycin resistant and possess a minimal inhibitory concentration of $\geq$160 μg/ml. In many of the isolates, the genetic location of the antibiotic resistance genes was shown to be present on conjugative plasmids.

Key words: Northern bobwhite, *Enterococcus*, multiple antibiotic resistance

INTRODUCTION

Organisms belonging to the genus *Enterococcus* are ubiquitous in nature, being found in very diverse environmental habitats as well as animal, bird, and invertebrate hosts (Dutka and Kawn, 1978; Kibby *et al*., 1978; Devriese *et al*., 1992a, 1992b; Blaimont *et al*., 1995). Currently five groupings, comprising sixteen different species are recognized (Facklam *et al*., 1999). Clinically, the majority of human infections associated with enterococci are due to *E. faecalis* and to a lesser extent *E. faecium* (Bryce *et al*., 1991; Guiney and Urwin, 1993). Other enterococcal species are less frequently, if at all, isolated clinically (Facklam and Collins, 1989; Facklam *et al*., 1999). However, these organisms, depending on their environmental origin, may possess various virulence and antibiotic resistance determinants that could be transferred to other clinically relevant species.

Enterococci have established themselves as important clinical pathogens in recent years. Oftentimes, these organisms carry a variety of antibiotic resistance genes, which make treatment with antibiotics difficult (Murray, 1991; Spera and Farber, 1994). Due to the widespread distribution of these organisms, reports documenting encounters with multiple drug resistant enterococci are increasing. For example, food animals harboring multiple antibiotic resistant enterococci are becoming more prevalent (Thal *et al*., 1995; Aarestrup *et al*., 2000) and may be serving as reservoirs of drug resistant enterococci. Ingestion of food products containing these organisms may allow transmission of resistance genes to clinically significant enterococci.

The Northern Bobwhite (*Colinus virginianus*) is a common game bird on the decline throughout the United States. Many game farms raise these birds to augment natural populations and provide a commercially available game bird or food source. These birds, like all other domestic or wild avian species, may serve as a reservoir for multiple drug resistant bacteria that can be transferred to other animals or humans upon contact, handling and/or consumption. However, information regarding the normal bacterial flora associated with Northern Bobwhite is lacking. As a result, our objective in this study was to determine the species distribution of enterococci associated with the intestinal tract of Northern Bobwhite and investigate their susceptibility to a panel of seven different antibiotics. Our results indicate the enterococcal flora is diverse and multiple drug resistance is widespread among isolates.

MATERIALS AND METHODS

Isolation and identification of *Enterococcus* species: Newly hatched to seven-day-old Northern Bobwhite chicks (n = 39) that had been fed a commercial game bird feed in captivity were obtained from Tobler's Game Birds, Lebo, Kansas, USA. There was no antibiotic use in these organisms prior to

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euthanasia. Chicks were euthanatized using 2-bromo-2-chloro-1,1,1-trifluoroethane (Sigma, St. Louis, Mo, USA). An intestinal fragment was removed and homogenized in 1 ml of sterile saline. One-tenth milliliter aliquots of serially diluted homogenate were spread plated on Bile Esculin Agar (BEA). After incubation overnight at 37°C, plates were examined for growth. Colonies appearing black in color were isolated and re-inoculated onto a fresh Tryptic Soy Agar (TSA) plate. Gram-positive organisms arranged in short chains were selected. Growth at 45°C, in 6.5% NaCl, the ability to hydrolyze leucine-ß-naphthylamide (LAP) and a negative catalase test confirmed the isolates belonged to the genus *Enterococcus* as previously indicated (Facklam *et al*., 1999). Confirmed enterococci were further subjected to routine biochemical testing used to identify individual species in the genus *Enterococcus* (Facklam *et al*., 1999). The ability to produce acid in broths containing the carbohydrates sorbose and mannitol were initially performed. Based on results obtained, growth with various other carbohydrates was investigated to identify the isolate at the species level.

Antimicrobial susceptibility testing: Each isolate was tested for susceptibility to seven different antibiotics using the disk diffusion method according to guidelines set by the National Committee for Clinical Laboratory Standards (NCCLS) (Anonymous, 1997; 1998a; 1998b; 1999). Briefly, organisms were propagated in Mueller-Hinton (MH) broth at 37°C with shaking at 250 rpm for approximately 16 h. A fresh MH broth was inoculated with 10 µl of the established culture and incubated until growth paralleled a 0.5 McFarland turbidity standard. Subsequently, a MH agar plate was surface inoculated using a sterile swab dipped into this culture. Antibiotic disks impregnated with ampicillin (10 µg), ciprofloxacin (5 µg), clindamycin (2 µg), erythromycin (15 µg), nitrofurantoin (300 µg), tetracycline (30 µg) and vancomycin (30 µg) were placed on the surface followed by incubation at 37°C for 24 h. Using NCCLS guidelines, each organism was classified as resistant or susceptible to each antibiotic based upon the zone of inhibition around each disk. Specifically, organisms were considered resistant if the diameter of the zone of inhibition was equal to or less than 16 mm for ampicillin, 15 mm for ciprofloxacin and clindamycin, 14 mm for nitrofurantoin, tetracycline and vancomycin and 13 mm for erythromycin. Antibiotic disks were purchased from Becton Dickinson Microbiology Systems (Cockeysville, MD, USA).

Isolates demonstrating resistance to vancomycin were further examined using a broth dilution assay to determine the Minimal Inhibitory Concentration (MIC). Briefly, vancomycin resistant isolates as determined via the disk diffusion assay were propagated in MH broth containing variable amounts of vancomycin. Following incubation at 37°C with shaking at 250 rpm for 24 h, the MIC was determined by visual inspection.

Transformation of antibiotic resistance via conjugation: Conjugation experiments were performed using each enterococcal isolate as the donor and *E. faecalis* JH2-2 as the recipient. All test isolates were rifampicin sensitive and tetracycline resistant, whereas *E. faecalis* JH2-2 was resistant to rifampicin and sensitive to tetracycline. Furthermore, *E. faecalis* JH2-2 was sensitive to all antibiotics examined in this study by the disk diffusion method described above (data not shown). Briefly, each test isolate and *E. faecalis* JH2-2 was propagated overnight in MH broth at 37°C with shaking at 250 rpm. Three hundred microliter was used to inoculate a fresh MH broth followed by incubation until a 0.5 McFarland turbidity standard was reached (determined visually). Five hundred microliter of each McFarland standardized test isolate was mixed with 500 µl of McFarland standardized *E. faecalis* JH2-2 culture. This combination of cells was mixed gently, incubated at 37°C for 90 min and 200 µl subsequently plated on MH agar plates containing rifampicin (25 µg/ml) and tetracycline (16 µg/ml). Plates were incubated overnight at 37°C to select for *E. faecalis* transconjugants.

RESULTS

Species distribution: Eighty-nine random enterococcal isolates were obtained from the intestine of Northern Bobwhite chicks and their species determined through biochemical testing. The species distribution of the isolates as well as phenotypic characteristics leading to their identification is outlined in Table 1.

Antibiotic sensitivity: Using the disk diffusion method, isolates were examined for susceptibility to seven different antibiotics and classified as resistant or susceptible according to NCCLS guidelines. As indicated in Table 2, all isolates demonstrated resistance to a minimum of two different antibiotics. Sixty-nine (78%) of the isolates were resistant to three different antibiotics, while sixteen (18%) of the isolates were resistant to four of the seven antibiotics examined. Due to the significance of vancomycin resistance among clinical *Enterococcus* isolates, we further determined via a broth dilution assay the MIC of vancomycin on those isolates determined to be vancomycin resistant by the disk diffusion method. All isolates were capable of growing in MH broths supplemented with 160 µg/ml vancomycin (data not shown). Growth with vancomycin concentrations higher than 160 µg/ml was not determined.

Conjugational transfer of antibiotic resistance: The ability of each *Enterococcus* isolate to confer an antibiotic resistance phenotype via conjugation to a

Table 1: Species distribution of *Enterococcus* Isolates obtained from Northern bobwhite quail

Organism/number of isolates[a]	Identifying biochemical properties
E. saccharolyticus/ 42 (47%)	sorbose (+); mannitol (+); arabinose (-); pyruvate (-)
E. malodoratus/ 20 (23%)	sorbose (+);mannitol (+); arabinose (-); pyruvate (+)
E. pseudoavium/ 8 (9%)	sorbose (+);mannitol (+); arabinose (-); raffinose (-)
E. raffinosus/ 7 (8%)	sorbose (+);mannitol (+); arabinose (-); raffinose (+)
E. faecalis/ 6 (7%)	sorbose (-);mannitol (+); pyruvate (+); tellurite (+)
E. mundtii/ 2 (2%)	sorbose (-);mannitol (+); arabinose (+); raffinose (+); motility (-); pigment (+)
E. avium/ 2 (2%)	sorbose (+);mannitol (+); arabinose (+); raffinose (-)
E. cecorum/ 1 (1%)	sorbose (-);mannitol (-); pyruvate (+); sorbitol (+)
E. durans/ 1 (1%)	sorbose (-);mannitol (-); arginine (+); sucrose (-)

[a]The number in parentheses represents the total percentage of the isolates belonging to the species indicated

Table 2: Antibiotic resistance patterns of *Enterococcus* species isolated from Northern bobwhite quail

Organism[a]		Resistance[b]	Organism[a]		Resistance[b]
E. saccharolyticus	(36)	T,Cl,E,	*E. raffinosus*	(6)	T,Cl,E
	(3)	T,Cl		(1)	T,Cl
	(1)	CP,T,Cl	*E. faecalis*	(5)	V,T,Cl,E
	(1)	V,T,Cl		(1)	T,Cl,E
	(1)	V,T,Cl,E	*E. mundtii*	(2)	T,Cl,E
E. malodoratus	(19)	T,Cl,E	*E. avium*	(1)	T,Cl,E
	(1)	V,Cl,E		(1)	V,T,Cl,E
E. pseudoavium	(8)	V,T,Cl,E	*E. cecorum*	(1)	T,Cl,E
E. durans	(1)	V,T,Cl,E			

[a]The number in parentheses beside or below each organism indicates the number of organisms with the resistance pattern indicated.
[b]Resistance pattern obtained by the disk diffusion assay. V, Vancomycin; Cl, Clindamycin; CP, Ciprofloxacin; T, Tetracycline; E, Erythromycin

Table 3: Antibiotic resistance of *Enterococcus faecalis* upon conjugational transfer from isolated *Enterococcus* species

Ogranism[a]		Wild type[b]	Transconjugant[b]
E. saccharolyticus	(15)	T,Cl,E	T,Cl,E
	(4)	T,Cl,E	T,Cl
	(2)	T,Cl	T,Cl
	(1)	Cp,T,Cl	T,Cl
E. malodoratus	(11)	T,Cl,E	T,Cl,E
	(1)	T,Cl,E	T,Cl
E. raffinosus	(5)	T,Cl,E	T,Cl,E
	(1)	T,Cl,E	T,Cl
E. mundtii	(1)	T,Cl,E	T,Cl,E
E. avium	(1)	T,Cl,E	T,Cl,E
E. cecorum	(1)	T,Cl,E	T,Cl,E

[a]The number in parentheses beside or below each organism indicates the number of organisms with the resistance pattern indicated.
[b]Resistance pattern obtained by the disk diffusion assay. V, Vancomycin; Cl, Clindamycin; CP, Ciprofloxacin; T, Tetracycline; E, Erythromycin

recipient *E. faecalis* host organism was investigated. As summarized in Table 3, 48% of the total isolates were able to transfer conjugable plasmids. In all experiments, those strains unable to transfer an antibiotic resistance phenotype during the initial conjugation experiment were also unable to do so when the experiment was repeated (data not shown). Examination of the majority of transconjugants revealed their antibiotic resistance pattern was the same as the wild type strain. When exceptions occurred, the inability to transfer erythromycin resistance was most notable.

DISCUSSION

We report the isolation, identification and antibiotic susceptibility of the intestinal enterococcal flora associated with Northern Bobwhite. All eighty-nine isolates were obtained from the culturing of homogenized intestinal fragments on BEA. Following genus confirmation as *Enterococcus*, a detailed biochemical analysis according to Facklam *et al*. (1999) delineated the isolates down to the species level. Our results indicate the species distribution associated with Northern Bobwhite appears to be quite distinct from that of similar studies in other bird species, such as turkeys (Welton *et al*., 1998), chickens (Joseph *et al*., 2001) and pigeons (Baele *et al*., 2002). In fact, an analogous study investigating caecum-associated enterococci in Japanese Quail revealed *E. gallinarum*, *E. avium* and *E. faecium* were predominant (Laukova *et al*., 1995). In comparison, our findings demonstrated *E. saccharolyticus* was the dominant *Enterococcus* species, whereas no *E. gallinarium* or *E. faecium* were isolated and only one isolate of *E. avium* was obtained (Table 1). These observations illustrate the enterococcal flora is avian species dependent and/or a function of the food source and environment in which these birds were housed. Also, our Northern Bobwhite sample population ranged from freshly hatched to seven day old chicks. The enterococcal flora obtained were those species that colonized the intestinal tract rapidly after birth and the diversity may be altered in older birds. Similarly, natural populations of birds occupy a range of environmental conditions, which may have significant effects on its bacterial flora.

Antibiotic sensitivity testing of the eighty-nine isolates was in accordance with NCCLS standards (Anonymous 1997;1999) and was composed of the penicillin (ampicillin), macrolide (erythromycin) and quinolone (ciprofloxacin) families in addition to other miscellaneous antibiotics (clindamycin, nitrofurantoin, tetracycline, vancomycin). As indicated in Table 2, multiple drug resistance was prevalent to varying degrees in all isolates. Most significantly from a public health standpoint, nineteen of the isolates were shown to be vancomycin resistant. We further characterized the vancomycin resistant isolates by determining the Minimal Inhibitory Concentration (MIC) through a broth dilution assay. All isolates that were shown to be resistant to vancomycin via the disk diffusion assay had an MIC of ≥160 μg/ml vancomycin, which is well above clinically resistant levels according to NCCLS guidelines (data not shown). Although most of the vancomycin resistant isolates are not clinically significant, an interesting note is that five out of the six *E. faecalis* isolated were vancomycin resistant (Table 2). The remaining vancomycin resistant isolates are currently not recognized as human pathogens, but have the potential to transfer genes conferring this resistance to organisms of greater clinical significance (Arthur and Courvalin, 1993). This exchange of genetic information could in theory occur through conjugative transposons, pheromone-responsive plasmids, or other broad-host-range plasmids (Rice *et al*., 1995). Although we did not determine the molecular nature of the resistance in this study, most vancomycin resistance is due to VanA and VanB (Arthur and Courvalin, 1993). Indeed, high-level vancomycin resistance is not uncommon among enterococci. For example, a previous study reported MICs of vancomycin were >512 μg/ml for over 90% of enterococci isolated from poultry (Bustamante *et al*., 2003). A separate investigation demonstrated high level vancomycin resistance in enterococci isolated from poultry farms in Norway (Borgen *et al*., 2000). These authors found the VanA resistance determinant, which is responsible for high level vancomycin resistance, was very stable in a non-selective environment.

Conjugation experiments revealed approximately half of the wild type isolates could transfer their antibiotic resistance phenotype to *E. faecalis* JH2-2. Having antibiotic resistance genes located on the chromosome, non-conjugable plasmids, or on conjugative plasmids unable to transfer at 37°C could account for those strains unable to transfer their antibiotic resistance via conjugation, however, we have no data to support these hypotheses. Furthermore, it is possible a different recipient organism would facilitate conjugation in a greater number of the test isolates. Regardless, our experiments conclusively demonstrate conjugative transfer of antibiotic resistance genes among *Enterococcus* species is possible. Previous studies have also documented conjugal transfer of tetracycline and/or erythromycin resistance among enterococci (Teuber *et al*., 1996; Huys *et al*., 2004).

Multiple drug resistant enterococci have been isolated worldwide from clinical and veterinary hosts as well as foods (Klare *et al*., 1995). It has been recognized for decades that a correlation exists between human disease and food sources. With increased usage of antibiotics in agriculture, it is not unlikely that antibiotic supplements given to food animals could result in increased human bacterial flora with drug resistant phenotypes. The benefits of supplementing animal feeds with various growth enhancers and antibiotics are evident through healthy food-production animals; however, drawbacks include providing the selective pressure for developing drug resistant bacteria. It is critical the scientific community establish surveillance systems to monitor trends in antimicrobial resistance and also perform basic research to establish what agricultural practices result in increased bacterial drug resistance in the environment and animals. In the case of bobwhites, domestic birds are regularly propagated for release into the environment for dog training and hunting activities and provide an avenue for increasing drug resistance in the environment.

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