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Thirteen-lined Ground Squirrels (*Spermophilus tridecemlineatus*) Harbor Multiantibiotic-resistant Bacteria

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Whether wild-caught animals used for biomedical research carry antibiotic-resistant bacteria is not well studied. Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) are small mammals used to study hibernation. These animals are captured from the wild or are born in laboratory animal facilities to wild-caught mothers. Because microorganisms harbored by 13-lined ground squirrels may be pathogenic to their caretakers and other laboratory animals, learning more about antibiotic resistance in these animals could be useful. In this study, tetracycline- and chloramphenicol-resistant *Morganella morganii* and multidrug resistant *Stenotrophomonas maltophilia* were isolated from the ceca of four 13-lined ground squirrels. These findings support further study of antibiotic-resistant bacterial populations in wild-caught mammals used as laboratory models.

Abbreviations: LB, Luria-Bertani; MIC, minimum inhibitory concentration; CFU, colony-forming units

The 13-lined ground squirrel (*Spermophilus tridecemlineatus*) is widely used in hibernation research, including studies on basic biology as well as those focused on biomedical applications of hibernation to human health.[4,5,15] The National Human Genome Research Institute recently completed an initial genome sequence project for this species; these results will further enhance the 13-lined ground squirrel's utility in research relating hibernation to biomedicine and other nonmedical investigations. Currently, most 13-lined ground squirrels in laboratory animal facilities were caught in the wild or were born to wild-caught mothers, though captive breeding programs are under development.[24] Concerns about wild-caught laboratory animals include post-capture mortality,[24] genotypic variation, unknown nutrition status, and variations in microflora. Little is known about the commensal bacteria in these animals,[2,3] including whether 13-lined ground squirrels harbor antibiotic-resistant bacteria. The commensal microbial communities of other laboratory animals contain antibiotic-resistant bacteria, such as antibiotic-resistant *Escherichia coli* in laboratory rats, hamsters, minipigs, rabbits, and mice.[9,22] Antibiotic-resistant bacteria also have been detected in multiple species of wild mammals and vary by host species and the proximity of the animals to humans.[8,16,19,21] The present report presents characterization of the antibiotic-resistant bacteria from the 13-lined ground squirrel cecum, including 4 multidrug-resistant isolates. These findings support the need for further study addressing antibiotic-resistant bacteria harbored by wild-caught mammals used as laboratory models.

Materials and Methods

Animals. Procedures for squirrel collection, maintenance, and experimentation were approved by the University of Wisconsin–Madison Institutional Animal Care and Use Committee (protocol V1229). Squirrels used in this study were either collected from the wild (vicinity of Madison, WI) as adults or were born in the animal facility from pregnant females collected in May (referred to as 'pups' and euthanized 8 to 13 mo after birth). Those collected from the wild were treated with flea spray (Frontline, Merial, Duluth, GA) and 0.03 mg ivermectin (Phoenix Pharmaceutical, St Joseph, MO) to eliminate nematode parasites prior to entry into the animal facility. Adult squirrels and weaned pups were housed individually in conventional rodent cages and provided free access to water and rodent chow (5001, Purina, St Louis, MO) supplemented with sunflower seeds every other week during the active season. Adults were maintained in the animal facility on the chow diet for at least 4 wk before use in experiments. Summer (active) squirrels were studied from June to early August. Mothers not euthanized during the summer and pups were allowed to hibernate. Squirrels were implanted with radiotelemeters (MiniMitter, Bend, OR) to monitor activity state during hibernation. At least 4 wk after telemetry placement, hibernation was induced by moving the squirrels to a cold (4 °C), dark room. Food and water were removed after squirrels began regular bouts of torpor. Animals were euthanized by 5% isofluorane anesthesia followed by decapitation (for active squirrels) or rapid decapitation alone (for hibernating squirrels). The cecal tissue was snap-frozen in liquid nitrogen and stored at –80 °C until processing.

Bacterial culturing. Cecal tissue samples were thawed at 37 °C for 30 min. The ceca were dissected, the contents removed, and the lumen rinsed with 1.0 ml of sterile phosphate-buffered saline. Chemicals were purchased from Sigma Chemical Company (St Louis, MO) except for potassium chloride (ICN, Aurora, OH). Approximately one quarter of the cecal tissue was harvested, weighed, macerated with a sterile plastic pestle, suspended in 1.5 ml phosphate-buffered saline and sonicated for 1 min. Serial dilutions were prepared in phosphate-buffered saline from the cecal suspensions. Total bacterial populations were determined by plating the cecal suspensions on Luria-Bertani (LB) media (Fisher Scientific, Pittsburgh, PA) and incubating at 37 °C for 24 h. Minimal inhibitory concentrations (MIC) for amoxicillin, ampicillin, chloramphenicol, gentamicin, naladixic acid, streptomycin, and tetracycline were determined for all aerobic bacteria isolated

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Table 1. Characteristics of 13-lined ground squirrels sampled

Squirrel number	State	Sex[a]	Age[b] (mo)	Body mass at euthanization (g)	Days housed in animal facility
1830	Hibernating	Male	Adult	124	144
1853	Hibernating	Female	Adult	172	162
2317	Active	Female	8	Not recorded	254
2320	Hibernating	Pup	9	145	288
2323	Hibernating	Pup	10	149	288
2326	Active	Male	13	182	396
2331	Hibernating	Pup	9	131	281
2333	Active	Female	13	195	405
2335	Hibernating	Pup	9	141	280
2359	Hibernating	Pup	9	134	284
2363	Active	Female	Adult	206	345
2375	Active	Female	Adult	186	349
2392	Hibernating	Female	Adult	134	221
2393	Hibernating	Female	Adult	139	200
2403	Hibernating	Male	Adult	109	236
2404	Hibernating	Male	Adult	151	218
2407	Active	Female	Adult	193	319
2409	Hibernating	Male	Adult	138	202
2411	Hibernating	Male	Adult	148	204
2428	Hibernating	Male	Adult	139	206
2438	Hibernating	Female	Adult	124	197
2441	Hibernating	Female	Adult	103	204
2447	Active	Male	Adult	186	310

[a]'Pup' indicates that the animal was euthanized before secondary sexual characteristics were recorded.
[b]'Adult' indicates that the animal was born in the wild and that its exact age is unknown.

from 2 additional ceca prior to beginning this study (data not shown). These values were used to determine the concentration of each antibiotic used for selection. Antibiotic resistant isolates were selected by plating cecal suspensions on LB containing 32 mg/l ampicillin, 32 mg/l amoxicillin, 10 mg/l chloramphenicol, 4 mg/l gentamicin, 32 mg/l naladixic acid, 10 mg/l streptomycin, or 16 mg/l tetracycline. After incubation, colonies were counted to estimate the number of colony-forming units per gram of tissue (CFU/g), and isolated bacteria were streaked on MacConkey agar (Difco, Franklin Lakes, NJ) plates.

Estimation of MIC. For each isolate selected by culture-based methods, the MIC for the antibiotic used for selection was measured. Each isolate was grown overnight with aeration at 37 °C. Saturated cultures were diluted 1:1000 in Mueller-Hinton broth (Becton Dickinson, Franklin Lakes, NJ). We added 2 µl of culture to 200 µl of Mueller-Hinton broth containing antibiotic in microtiter plates, which were incubated at 37 °C without aeration; growth in each well was observed after 24 h. MIC was defined as the lowest concentration of antibiotic at which no bacterial growth occurred.

Confirmation of MIC for Enterobacteriaceae. The MIC for each antibiotic was confirmed following standard clinical laboratory procedures for broth microdilution.[11] Briefly, each isolate was grown on LB agar for 16 to 18 h. Isolated colonies were suspended in Mueller-Hinton broth, and the culture density was adjusted to approximately 1×10^8 CFU/ml by measuring optical density at 600 nm and comparing this value with a dilution curve for each isolate. Each suspension was diluted 1:20 in Mueller-Hinton broth, and 10 µl was used to inoculate 100 µl of Muller-Hinton broth containing a dilution series of the test antibiotic in 96-well plates. The concentration and purity of each inoculum was assessed by plating a dilution series on LB agar. Both the MIC plates and LB agar plates were incubated for 16 to 18 h at 36 °C. The MIC recorded was the lowest concentration of antibiotic to inhibit +3 or +4 growth (heavy turbidity or granular appearance in well) based on visual assessment and comparison with uninoculated wells, inoculated wells containing only Mueller-Hinton broth, and each antibiotic dilution series inoculated with *Escherichia coli* DH10B or *E. coli* DH5α. MIC values were measured in 3 independent experiments for each isolate. Isolate numbers were not coded prior to interpretation.

Isolate identification. The 16S rRNA gene of each isolate was amplified by polymerase chain reaction (PCR) using the primers 27F (5' AGR GTT TGA TYM TGG CTC AG 3') and 1492R (5' GGY TAC CTT GTT ACG ACT T 3') at an annealing temperature of 55 °C.[14] PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) according to the manufacturer's instructions. Each PCR product was sequenced with 27F and 1492R primers and Big Dye 3.1 (Applied Biosystems, Foster City, CA). Reaction products were purified with CleanSeq (Agencourt Bioscience, Beverly, MA) according to the manufacturer's instructions. Results were analyzed on automatic sequencers (3730xl, Applied Biosystems) at the University of Wisconsin DNA Sequencing Facility. Sequences obtained were compared with those in GenBank by use of the Basic Local Alignment Search Tool.[1] Physiologic confirmation of Enterobacteriaceae isolates was performed using API 20E strips (bioMerieux, Marcy l'Etoile, France) according to the manufacturer's instructions. *Morganella morganii* isolates were tested for trehalose utilization on bromcresol purple agar (10 g/l peptone, 5 g/l NaCl, 3 g/l beef extract, 0.04 g/l bromcresol purple, 5 g/l trehalose, and 15 g/l agar) to identify subspecies.

Results

The ground squirrels used in this study included 5 pups, 8 adult males, and 10 adult females, of which 16 were hibernating and 7 were active (Table 1). The total population of bacteria cultured

Table 2. Antibiotic-resistant bacteria isolated from 13-lined ground squirrels

Isolate	Organism	Antibiotic-resistance phenotypes (minimal inhibitory concentration; µg/ml)						
		Ampicillin	Amoxicillin	Chloramphenicol	Gentamicin	Nalidixic acid	Streptomycin	Tetracycline
SQ249	*Morganella morganii*	**512**	**>512**	**32**	2	4	16	32
SQ314	*Morganella morganii*	**512**	**512**	**32**	2	4	4	**128**
SQ331	*Morganella morganii*	**512**	**>512**	**32**	2	4	4	**32**
SQ359	*Stenotrophomonas maltophilia*[a]	>512	>512	**64**	**>128**	16	512	**128**

Boldface type indicates a minimal inhibitory concentration equal to or greater than the value for clinical resistance to the specified antibiotic in Enterobacteriaceae (ampicillin, 32 µg/ml; amoxicillin, 32 µg/ml; chloramphenicol, 32 µg/ml; gentamicin, 16 µg/ml; nalidixic acid, 32 µg/ml; streptomycin, 32 µg/ml; tetracycline, 16 µg/ml).[18]

[a]Testing procedures for measuring antibiotic resistance in *S. maltophilia* vary.[6,7,10,12] The values reported were obtained following the broth microdilution protocol used for Enterobacteriaceae isolates. Clinical resistance values for *S. maltophilia* are: chloramphenicol, 32 µg/ml; gentamicin, 16 µg/ml; and tetracycline, 16 µg/ml.[18]

aerobically on LB agar from each sample of cecal tissue ranged from 8.1×10^2 to 7.1×10^6 CFU/g, and the number of distinct bacterial morphologies in each sample ranged from 2 to 13.

We further screened 85 isolates selected on LB plus antibiotic to assess whether they demonstrated MICs equal to or above the breakpoints for clinical isolates of the Enterobacteriaceae. The gram-negative bacteria screened were predominantly γ-*Proteobacteria.* These isolates included *Providencia rettgeri*, *Morganella morganii*, *Escherichia fergusonii*, *Proteus* sp., *Pseudomonas* sp., *Stenotrophomonas maltophilia*, and *Comamonas* sp. A greater phylogenetic diversity of gram-positive bacteria was selected on the antibiotic-containing media. Isolates screened included *Staphylococcus* sp., *Enterococcus* sp., *Bacillus* sp., *Paenibacillus* sp., and *Chryseobacterium* sp. Antibiotic resistance is a single term encompassing multiple phenotypes. Bacteria can be inherently resistant to an antibiotic because they lack the drug target or compatible physiology for activity. Alternatively, they may have acquired resistance through mutation or mobile DNA elements.[17] Not surprisingly, in most cases, the antibiotic resistance patterns observed in bacterial isolates from the 13-lined ground squirrels were characteristic for the species identified; however, some nonintrinsic antibiotic resistance was seen.

Isolates exhibiting antibiotic resistance profiles that were atypical for their taxonomic classification were characterized further. MIC values were confirmed in 3 separate experiments that followed recommended clinical laboratory practices, and the classification of the isolates was verified by physiological testing. The identities and MIC information for each antibiotic resistant isolate are listed in Table 2.

Three strains of *Morganella morganii* resistant to tetracycline and chloramphenicol were isolated from squirrels 2403, 2438, and 2441. The *M. morganii* isolates from squirrels 2438 and 2441 were subspecies *sibonii*, whereas the isolate from squirrel 2403 was subspecies *morganii*.[23] The number of *M. morganii* found ranged from 1×10^2 to 5×10^2 CFU/g. *Stenotrophomonas maltophilia* (SQ359) was isolated from squirrel number 2326 at a population of 2×10^3 CFU/g. SQ359 was resistant to all antibiotics tested at an intermediate or high level (Table 2).

Discussion

In this study, we demonstrated that 13-lined ground squirrels harbor bacteria that are resistant to a broad spectrum of antibiotics. Several pieces of data suggest that the multiantibiotic-resistant bacteria present in the cecal samples were residents of the squirrel commensal microbial community and not transient contaminants obtained after the squirrels entered the animal facility. It is noteworthy that many of the bacteria were isolated from animals in hibernation (and therefore were not eating). In addition, bacteria with similar antibiotic resistance patterns were not isolated from the ceca of 3 female, 2-mo-old Balb/C mice untreated with antibiotics and housed in the same animal facility or from a female, ICR mouse housed in the same room and under the same conditions as the active squirrels for 82 d (data not shown). The 4 isolates recovered are likely an underestimate of the overall frequency of antibiotic-resistant bacteria in the cecal microbial community. Bacteria could have been destroyed during freezing, thawing, or sonication of the cecal tissue. In addition, the culture techniques used would prevent detection of obligate anaerobic bacteria and organisms unable to grow on standard laboratory media, such as fastidious organisms requiring serum supplementation.

The results presented here suggest that investigation of the potential for transfer of antibiotic-resistant and pathogenic bacteria from 13-lined ground squirrels to humans is warranted. Although *S. maltophilia* is a nosocomial human pathogen, the reservoirs of this organism are not well understood.[20] In addition, *M. morganii* ssp. *morganii* can infect humans.[23] Both of these isolates were identified in the normal microbial communities of 13-lined ground squirrels. Transfer of a pathogenic strain of *Bartonella washoensis* between California ground squirrels (*S. beecheyi*) and a cardiac patient has been reported;[13] antibiotic-resistant or pathogenic bacteria potentially could be transferred in the same fashion between 13-lined ground squirrels and humans or other animals. Understanding more fully the commensal bacterial communities of laboratory animals of wild origin may better inform their caretakers about potential health risks.

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References

1. **Altschul, SF, Gish, W, Miller, Myers, EW, Lipman, DJ.** 1990. Basic local alignment search tool. J Mol Biol **215:**403–410.
2. **Barnes EM, Burton GC.** 1970. The effect of hibernation on the caecal flora of the thirteen-lined ground squirrel (*Citellus tridecemlineatus*). J Appl Bact **33:**505–514.
3. **Blossman-Myer BL, Stangl FB Jr.** 1999. Fecal analysis as an indicator of post-hibernation shifts in the gut microflora of the thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*). Texas J Sci **51:**159–172.

4. **Carey HV, Andrews MT, Martin SL.** 2003. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiol Rev **83:**1153–1181.
5. **Carey HV, Southard JH, Mangino MJ.** 2001. Changes in gut function during hibernation—implications for bowel transplantation and surgery. Gut **49:**459–461.
6. **Dawis MA, Isenberg HD, France KA, Jenkins SG.** 2003. In vitro activity of gatifloxacin alone and in combination with cefepime, meropenem, piperacillin, and gentamicin against multidrug-resistant organisms. J Antimicrob Chemother **51:**1203–1211.
7. **Friedman ND, Korman TM, Fairley CK, Franklin JC, Spelman DW.** 2002. Bacteraemia due to *Stenotrophomonas maltophilia*: an analysis of 45 episodes. J Infect **45:**47–53.
8. **Gilliver MA, Bennett M, Begon M, Hazel SM, Hart CA.** 1999. Antibiotic resistance found in wild rodents. Nature **401:**233–234.
9. **Hansen AK, Velschow S.** 2000. Antibiotic resistance in bacterial isolates from laboratory animal colonies naive to antibiotic treatment. Lab Anim **34:**413–422.
10. **Hejnar P, Hamal P, Koukalova D.** 2000. Influence of an extended incubation period on values of minimum inhibitory concentrations of antibiotics in *Stenotrophomonas maltophilia* clinical strains. New Microbiol **23:**415–421.
11. **Hindler JF, Tamashiro L.** 2004. Broth microdilution MIC test. In: Clarke L, Della-Latta P, Denys GA, Douglas SD, Garcia LS, Hazen KC, Hindler JF, Jenkins SG, Mangels JI, Miller JM, Nachamkin I, Pfaller MA, Snyder JW, Weissfeld AS, York MK, editors. Clinical microbiology procedures handbook. Washington (DC): ASM Press. p 5.2.1–5.2.17.
12. **King, A.** 2001. Recommendations for susceptibility tests on fastidious organisms and those requiring special handling. J Antimicrob Chemother **48(Suppl 1):**77–80.
13. **Kosoy M, Murray M, Gilmore RD Jr, Bai Y, Gage KL.** 2003. *Bartonella* strains from ground squirrels are identical to *Bartonella washoensis* isolated from a human patient. J Clin Microbiol **41:**645–650.
14. **Lane DJ.** 1991. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics. New York: John Wiley & Sons. p 115–175.
15. **Lindell SL, Klahn SL, Piazza TM, Southard JH, Carey HV.** 2005. Natural resistance to liver cold ischemia-reperfusion injury associated with the hibernation phenotype. Am J Physiol Gastrointest Liver Physiol **288:**G473–G480.
16. **Livermore DM, Warner M, Hall LMC, Enne VI, Projan SJ, Dunman PM, Wooster SL, Harrison G.** 2001. Antibiotic resistance in bacteria from magpies (*Pica pica*) and rabbits (*Oryctolagus cuniculus*) from west Wales. Environ Microbiol **3:**658–661.
17. **McDermott PF, Zhao S, Wagner DD, Simjee S, Walker RD, White DG.** 2002. The food safety perspective of antibiotic resistance. Anim Biotechnol **13:**71–84.
18. **National Committee for Clinical Laboratory Standards.** 2004. Performance standards for antimicrobial susceptibility testing: fourteenth informational supplement. Wayne (PA): NCCLS.
19. **Osterblad M, Norrdahl K, Korpimaki E, Huovinen P.** 2001. How wild are wild mammals? Nature **409:**37–38.
20. **Senol E.** 2004. *Stenotrophomonas maltophilia*: the significance and role as a nosocomial pathogen. J Hosp Infect **57:**1–7.
21. **Sherley M, Gordon DM, Collignon, PJ.** 2000. Variations in antibiotic resistance profile in Enterobacteriaceae isolated from wild Australian mammals. Environ Microbiol **2:**620–631.
22. **Shimoda K, Kimura J, Oguri S, Nakamura N, Urano T, Maejima K.** 2002. Penetration of drug resistance in *Escherichia coli* isolated from laboratory animals. J Vet Med Sci **64:**1133–1135.
23. **Stock I, Wiedemann B.** 1998. Identification and natural antibiotic susceptibility of *Morganella morganii*. Diagn Microbiol Infect Dis **30:**153–165.
24. **Vaughan DK, Gruber AR, Michalski ML, Seidling J, Schlink S.** 2006. Capture, care, and captive breeding of 13-lined ground squirrels, *Spermophilus tridecemlineatus*. Lab Anim **35:**33–40.