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Chlortetracycline-Resistant Intestinal Bacteria in Organically Raised and Feral Swine[▽]

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Organically raised swine had high fecal populations of chlortetracycline (CTC)-resistant (growing at 64 μg CTC/ml) *Escherichia coli*, *Megasphaera elsdenii*, and anaerobic bacteria. By comparison, CTC-resistant bacteria in feral swine feces were over 1,000-fold fewer and exhibited lower taxonomic diversity.

To examine the tetracycline resistance properties (MIC values and class genotype) of fecal bacteria cultured from swine which had never been exposed to commercially produced antibiotics, organically raised and feral swine were investigated. Ten grower-phase swine (40 to 50 kg, 10 to 12 weeks old), mixed sexes, from two organic farms, were housed and fed as described previously (19). Freshly voided fecal samples ("catch samples") were obtained weekly from each animal, and two or three samples were processed. Fecal samples from 19 feral swine were collected during two trips to Hobcaw Barony, a 17,000-acre, wildlife/forestry preserve on a peninsula distant from agricultural lands and near Georgetown, SC (20). The pigs were mixed sexes, 13.5 to 41 kg, and were 1 to 4 years old, as estimated from tooth structure. Based on previous studies (24) and our own observations of gut contents, the diet of these animals was primarily acorns but also grass, wild rice, leaves, plant roots, invertebrates, and mushrooms. The swine were killed within 12 h of trapping, and distal colon-rectal contents were obtained. Five-gram samples were deposited into large Hungate-type anaerobic tubes containing 35 ml of anaerobic heart infusion (HI) broth under nitrogen gas (18). The tubes were tightly sealed with rubber stoppers and plastic tape, packed in wet ice, and transported within 36 h to the National Animal Disease Center (NADC).

Intestinal bacteria were isolated on chlortetracycline (CTC)-containing culture media so that their *tet* gene content could be determined and associated with their taxonomic identity. RTC is a clarified rumen fluid-based medium, containing Trypticase-peptone and carbohydrates, and is used for general-purpose isolation of diverse intestinal bacteria (1, 18). Me109M is a simple culture medium designed for the selective isolation of *Megasphaera elsdenii* (19).

In a Coy anaerobic chamber, fecal samples were blended and serially diluted in HI broth, and the dilutions were spread-plated onto RTC and MacConkey agar media containing different concentrations of chlortetracycline (Tables 1 and 2). Serial dilutions were also plated onto Me109M plates without chlortetracycline to obtain *Megasphaera elsdenii* isolates for use in a patch test for CTC resistance (18a). RTC (total anaerobes) and Me109M plates were incubated anaerobically and MacConkey plates (*E. coli*) were incubated aerobically, at 39°C.

Estimated total populations of cultivable fecal anaerobic bacteria from organically raised swine averaged 5.9×10^{10} CFU/g feces (Table 1). These levels are consistent with previously reported cultivable bacterial concentrations in feces and intestinal contents of conventional swine (1, 18). Surprisingly, total anaerobe populations in feral swine were 60-fold lower, 1.1×10^{9} CFU/g feces (Table 1). The lower numbers of cultivable bacteria in samples from feral swine were consistent with lower optical turbidities of fecal sample dilutions. There were also fewer microscopically visible bacteria for feral sample dilutions than for those from organic swine. To rule out the possibility that lower viable bacterial counts of feral swine resulted from viability losses during the 36-h transport of the samples at 4°C, cultivable bacterial populations for nine feral swine were determined pretransport, by using an anaerobic roll tube technique (7). The roll tube method gave a pretransport average anaerobe level of 8.1×10^{8} CFU/g (standard error of the mean [SEM] = 2.9×10^{8}). For the same samples, post-transport viable counts averaged 8.6×10^{8} CFU/g (SEM = 1.4×10^{8}). Based on insignificant differences ($P > 0.9$, confidence interval [CI] = 95%, paired *t* test) due to transport, the feral swine in these studies had lower numbers of cultivable bacteria in their intestinal tracts than did organically raised swine. A previous report (24) and our own observations of gut contents indicated that the diet of these feral animals was primarily acorns but also grass, wild rice, leaves, plant roots, invertebrates, and mushrooms. A subsistence diet and high parasite load (diverse and abundant helminth species detected in animal tissues and organs at necropsy) likely contribute both to the small body size of the feral swine and to the reduced population levels of their intestinal microbiota.

Fecal anaerobic bacteria able to grow on media containing 64 μg and 256 μg CTC/ml were present in organic swine feces at estimated concentrations of 9.5×10^{9} and 1.4×10^{8} CFU/g, respectively (Table 1). In contrast, feral swine tetracycline-

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TABLE 1. Chlortetracycline-resistant anaerobic bacterial populations in fecal samples from organically raised and feral swine[a]

Swine origin	Total anaerobe population levels at chlortetracycline concn (μg/ml)[b]:		
	0	64	256
Organically raised	5.9×10^{10} (A)	9.5×10^{9} (16%) (B)	1.4×10^{8} (0.24%) (C)
Feral	1.1×10^{9} (A)	3.2×10^{6} (0.3%) (B)	$<2.6 \times 10^{4}$ (<.003%) (C)

[a] Values in the table are the average viable population levels (expressed as CFU/g [wet weight] of feces) of bacteria cultured anaerobically on RTC agar medium (spread plate method) and containing different concentrations of chlortetracycline.

[b] Anaerobe populations on RTC medium without chlortetracycline for 10 organically raised swine ranged from to 2.9×10^{10} to 9.1×10^{10} CFU/g (SEM = 7.4×10^{9}) and for 19 feral swine ranged from 2.5×10^{8} to 3×10^{9} CFU/g (SEM = 1.8×10^{8}). Values in parentheses represent chlortetracycline-resistant CFU as percentages of total CFU for each animal group. Values with similar uppercase letters in parentheses (A, B, and C) were significantly different ($P < 0.001$, CI = 95%, Welch's t test).

resistant anaerobe populations were significantly lower, 0.3% versus 16% at 64 μg CTC/ml and <0.003% versus 0.3% at 256 μg CTC/ml (Table 1).

Escherichia coli populations in feces from organically raised and feral swine were comparable (Table 2). Nevertheless, nearly 30% of *E. coli* cells cultured from organic swine were resistant to 64 μg CTC/ml. In contrast, feral swine contained undetectable levels of CTC-resistant *E. coli* (fewer than 5×10^{3} CFU/g feces).

M. elsdenii fecal populations averaged 2.4×10^{8} CFU/g in organically raised swine and were significantly lower, 1.5×10^{6} CFU/g, in feral swine samples (Table 3). Of 540 *M. elsdenii* isolates from organically raised swine, 52% were resistant to 64 μg CTC/ml (Table 3). In contrast, not one of 91 *M. elsdenii* isolates from feral swine was CTC resistant.

To identify *tet* genes in swine fecal isolates, 110 randomly selected strains (82 from organically raised and 28 from feral swine) resistant to 64 μg CTC/ml were cloned by subculturing isolated colonies twice and analyzed in PCR assays targeting classes *tet*(A) to *tet*(E), *tet*(G), *tet*(H), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(T), *tet*(W), and *tet*(36) and recombinant *tet*(O)-*tet*(W) genes (2, 3, 18, 19, 23). The sequence of the *rrs*-V3 region was used to differentiate isolates and to assess taxonomic affiliation (12, 18).

TABLE 2. Chloretracycline-resistant *E. coli* populations in fecal samples from organically raised and feral swine[a]

Swine origin	*E. coli* population at chlortetracycline concn (μg/ml):		
	0	16	64
Organically raised	6.1×10^{6}	4.9×10^{6} (73%) (A)	1.9×10^{6} (28%) (B)
Feral	2.0×10^{7}	<LOD (<.03%) (A)	<LOD (<.03%) (B)

[a] Values in the table are the average cultivable populations (expressed as CFU/g [wet weight] of feces) of lactose-fermenting bacteria cultured aerobically on MacConkey agar plates containing lactose (spread plate method). *E. coli* populations in 10 organically raised swine (6.1×10^{6}) were not significantly different ($P > 0.1$, CI = 95%, Welch's t test) from those of 19 feral swine (2.0×10^{7}). Values in parentheses represent chlortetracycline-resistant *E. coli* CFU expressed as percentages of total cultivable *E. coli* bacteria for each animal group. Percent values with the same uppercase letters in parentheses (A and B) were significantly different ($P < 0.001$, CI = 95%, Welch's t test). The limit of detection (LOD) was 5×10^{3} CFU/g feces for medium containing chlortetracycline.

TABLE 3. Chlortetracycline sensitivities of *Megasphaera elsdenii* isolates from organically raised and feral swine[a]

Swine origin	No. of *M. elsdenii* isolates growing at chlortetracycline concn (μg/ml):		
	0	16	64
Organic	540	355 (66%)	264 (52%)
Feral	91	0	0

[a] *M. elsdenii* population levels averaged 2.4×10^{8} CFU/g (range, 1.2×10^{8} to 4×10^{8}) in organically raised swine. *M. elsdenii* cultivable populations in feral swine were significantly lower than those in organically raised animals, averaging 1.5×10^{6} CFU/g ($P < .001$, CI = 95%, Welch's t test), and also more variable (range, $<5 \times 10^{3}$ to 2×10^{7} CFU/g). Chlortetracycline susceptibilities of *M. elsdenii* cloned isolates were determined by replica-patching colonies sequentially onto PYG agar plates containing 64, 16, and 0 μg chlortetracycline per ml (18a). Values in parentheses indicate resistant isolates expressed as percentages of totals tested.

Most (75% or 21/28) of the feral CTC-resistant taxa were *Lactobacillaceae* or *Streptococcaceae* (Table 4). These were not, however, the predominant taxa among the total feral bacterial isolates (unpublished observations). Resistant isolates from organic swine, by contrast, appeared taxonomically more diverse (Table 4).

Eight organic and three feral swine isolates did not have detectable *tet* genes (Table 4), suggesting that their resistance genes have minor sequence differences in regions targeted by the PCR primers (13) or represent a *tet* class not targeted in this study (5, 10, 21). Most CTC-resistant swine isolates (99/110) were positive in PCR assays for *tet* genes (Table 4). Their *tet* gene content was often consistent with that of related or identical taxa as described in the Antibiotic Resistance Genes Database (ARDB) (11). For example, *tet*(L), *tet*(M), and *tet*(O) have been found in *Streptococcaceae* (11). *E. coli* tetracycline-resistant strains can carry *tet*(A) or *tet*(B) (11), and both genes were detected in CTC-resistant *E. coli* from organic swine. At least one genus, *Lawsonia*, assigned to the *Desulfovibrionales* carries *tet*(W), and *Lawsonia* has been detected in feral swine feces (11, 15). CTC resistance genes, to our knowledge, have not been reported for taxa assigned to the *Coriobacteriaceae* and *Fusobacteriaceae* (11). Resistant isolates affiliated with those families were isolated from organically raised swine and were positive for *tet*(W) (Table 4). Mosaic (interclass recombinant) *tet*(O)-*tet*(W) genes were present in *M. elsdenii* isolates but were not detected in other anaerobes from the same organic swine (Table 4). Mosaic *tet* genes have been detected in fecal DNAs and bacterial strains from humans and swine (14, 21).

Each of two *Streptococcaceae* isolates, from organically raised swine, had two *tet* genes (Table 4). More recently, the genome of *M. elsdenii* strain 14-14, from an organic pig, was sequenced, and two genes, *tet*(40) and mosaic *tet*(OWO), were found in close proximity (T. B. Stanton and S. B. Humphrey, unpublished observations). Linkage between a mosaic *tet* gene and a nonmosaic *tet* gene has been noted for other intestinal bacteria (9, 21). Based on the above findings, other isolates listed in Table 4 could have additional *tet* genes, either duplicated genes or genes not detected by PCR assays.

Chlortetracycline is a common antibiotic used in swine feed

TABLE 4. Tetracycline resistance genotypes of fecal bacteria from organically raised and feral swine[a]

Swine origin	Tetracycline resistance gene class	No. of isolates tested	Taxonomic affiliation
Organic farm	Unk	2	*Actinobacteria-Bifidobacteriales*
	Unk	6	*Firmicutes-Selenomonadales*
	tet(A)	4	*Proteobacteria-Enterobacteriaceae*, *Escherichia coli*
	tet(B)	3	*Proteobacteria-Enterobacteriaceae*, *Escherichia coli*
	tet(L)	3	*Firmicutes-Streptococcaceae*[b]
	tet(M)	1	*Firmicutes-Streptococcaceae*
	tet(O)	1	*Firmicutes-Streptococcaceae*
	tet(O)	9	*Firmicutes-Clostridiales*
	tet(O)	3	*Firmicutes-Selenomonadales*
	tet(O)	1	*Firmicutes-Selenomonadales*, *Megasphaera elsdenii*
	tet(OW)	8	*Firmicutes-Selenomonadales*, *Megasphaera elsdenii*
	tet(OWO)	11	*Firmicutes-Selenomonadales*, *Megasphaera elsdenii*
	tet(W)	2	*Firmicutes-Selenomonadales*, *Megasphaera elsdenii*
	tet(W)	4	*Firmicutes-Selenomonadales*
	tet(W)	4	*Firmicutes-Clostridiales*
	tet(W)	2	*Firmicutes-Erysipelotrichaceae*
	tet(W)	7	*Firmicutes-Lactobacillaceae*
	tet(W)	5	*Actinobacteria-Coriobacteriaceae*
	tet(W)	1	*Fusobacteria-Fusobacteriales*
	tet(Q)	5	*Bacteroidetes/Chlorobi-Bacteroidales*
Feral	Unk	3	*Firmicutes-Lactobacillaceae*
	tet(M)	1	*Firmicutes-Streptococcaceae*
	tet(O)	17	*Firmicutes-Streptococcaceae*
	tet(B)	3	*Proteobacteria-Enterobacteriaceae*, *Escherichia coli*
	tet(W)	2	*Firmicutes-Clostridiales*
	tet(W)	2	*Proteobacteria-Desulfovibrionales*

[a] Tetracycline resistance gene class was determined from PCR assays. Unk, unknown, isolate was negative for all PCR assays. Except for *E. coli* and *M. elsdenii*, fecal bacteria were selectively isolated on RTC agar plus 64 μg CTC/ml (Table 1) and differentiated from each other based on *rrs*-V3 sequences. *E. coli* isolates were obtained from MacConkey agar medium plus 16 or 64 μg CTC/ml (Table 2). *Megasphaera elsdenii* isolates were selected from colonies able to grow on PYG agar medium containing 64 μg CTC/ml (Table 2) and include previously described strains (19).

[b] One *Streptococcaceae* isolate was positive in PCR assays for both *tet*(L) and *tet*(O) genes, and a second isolate was positive for both *tet*(L) and *tet*(M) genes.

both for the treatment and for the prevention of diseases that affect animal productivity (4, 6). Feral swine remote from contact with commercial antibiotic sources had relatively low bacterial populations in their intestinal tracts, and a low proportion of those were CTC resistant. We initially considered that studies of feral swine populations would provide baseline levels of "natural" resistance in swine, that is, levels useful for judging efforts to reduce the incidence of antibiotic resistance in farmed swine. However, the unexpectedly low populations of intestinal bacteria in the feral swine (likely attributable to poor diet and parasites) complicate this idea. At the same time, these results suggest that feral swine could prove useful experimentally, i.e., for examining the effects of commercial swine diets (with and without antibiotics) on a unique intestinal ecosystem with initially low antibiotic resistance.

In contrast to results for feral swine, a high proportion of total intestinal bacteria and *E. coli* and *M. elsdenii* isolates from organically raised swine were CTC resistant. The bacteria were taxonomically diverse and represented substantial numbers of tetracycline-resistant bacteria. In absolute numbers, at this concentration, an organically raised hog would shed an estimated 70 billion viable and tetracycline-resistant bacteria/day. For the sake of information, there are 16 million hogs (predominantly nonorganic) in Iowa.

These and other studies (summarized in reference 18a) have revealed that antibiotic-resistant populations and resistance genes persist in mammalian intestinal tracts even in the absence of direct antibiotic selection (8, 22). Although the organic farms had been free of antibiotic use for 4 years, the lineage of these animals had been exposed to more traditional farming practices (i.e., diets containing antibiotics). Baby pigs "inherit" or acquire their gut microbiota from the mother sows and were only several generations removed from their nonorganically raised progenitors.

While there is general agreement that antibiotics select for the development and growth of antibiotic-resistant bacteria, the basis for persistence of antibiotic resistance is unclear and may have multiple explanations, for example, the free exchange of antibiotic resistance genes among intestinal species or the continuous entry of resistance genes into the intestinal ecosystem through environmental sources, including feed (8, 16, 17). Another hypothesis is that subspecies diversity could contribute to the competitive fitness and maintenance of certain antibiotic-resistant intestinal species (18a). These findings suggest that approaches in addition to prudent antibiotic use will be important in effectively reducing resistant bacterial populations in swine.

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