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Detection of Tetracycline Resistance Determinants in Pig Isolates from Three Herds with Different Histories of Antimicrobial Agent Exposure

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A total of 114 gram-negative fecal isolates from domestic pigs in herds with different histories of antimicrobial agent exposure were screened for the presence of plasmid DNA and specific tetracycline resistance determinants. More than 84% of the isolates harbored plasmid DNA, which ranged in size from 2.1 to 186 kb. A total of 78 isolates (68.4%) were resistant to tetracycline at concentrations greater than 4 μg/ml. Plasmid DNAs from about 56% of the tetracycline-resistant isolates hybridized with DNA probes for class A, B, C, and D tetracycline resistance determinants. The class B determinant was the most common determinant (35% of the isolates), followed by the class C determinant (12%) and the class A determinant (1%). About 9% of the isolates contained two determinants on plasmids. None of the plasmids from isolates hybridized with the class D determinant probe. The class C determinant was the most prevalent determinant on plasmids in isolates from pigs not exposed to antimicrobial agents for more than 146 months, while the class B determinant was more prevalent on plasmids in isolates from pigs exposed to either subtherapeutic or therapeutic levels of antimicrobial agents. Most tetracycline resistance determinants were localized on plasmids which were more than 30 kb long. A great number of wild-type tetracycline-resistant *Escherichia coli* strains were found with the class E determinant on their chromosomes. This study revealed a high prevalence of tetracycline resistance determinants in the fecal flora of pig herds whether or not they were fed with antibiotics.

The use of antimicrobial agents in animal feeds for growth promotion, for improved feed efficiency, and for control and prevention of disease has been widespread since the early 1950s (11, 12, 15). Feeding subtherapeutic and therapeutic levels of tetracycline to pigs results in a high proportion of tetracycline-resistant microorganisms in the feces and intestinal tracts of swine (7, 16, 18, 29). Several reports (19, 20) have indicated that tetracycline resistance is widespread among bacterial species and can be accompanied by resistance to other structurally unrelated drugs. Tetracycline inhibits protein synthesis by interfering with the binding of the tRNA to the acceptor site of the ribosome (30). Three biochemical mechanisms of resistance have been reviewed by Salyers et al. (34). The efflux mechanism of tetracycline resistance is assumed to be present in the enteric bacteria (25).

In most cases, tetracycline resistance genes are carried on bacterial plasmids in both gram-negative and gram-positive species (5). A minimum of five classes of genetically distinguishable tetracycline resistance determinants, designated classes A through E, have been described in aerobic gram-negative enteric bacteria (22–24, 26). These determinants are not uniformly distributed, and they are often associated with specific genera and species.

Previous research (17) has shown that tetracycline was the predominant drug in the resistance pattern in fecal coliform bacteria isolated from pigs in three herds with different histories of antimicrobial agent exposure. The percentages of tetracycline-resistant isolates were approximately 26% for a herd that was not exposed to antimicrobial agents, 76% for a herd in which antimicrobial agents were used only for therapeutic purposes, and almost 100% for a herd which was continuously exposed to antimicrobial agents (17). Such resistant bacteria represent a constant pool of resistant genes that are potentially transferable to human pathogens.

The objectives of this study were to determine which classes of tetracycline resistance determinants were present in isolates from pigs and to compare the distribution of specific tetracycline resistance determinants in isolates from pigs in three herds with different histories of antibiotic exposure. To identify and localize these determinants, DNA-DNA hybridization experiments were performed with probes containing tetracycline resistance determinants belonging to classes A to E, O, and M.

MATERIALS AND METHODS

Bacterial strains. The 114 wild-type gram-negative enteric isolates used in this study were isolates recovered during other studies involving pigs belonging to three herds with different histories of antimicrobial agent exposure (17). The antibiotic-exposed (AB), non-antibiotic-exposed (NAB), and therapeutic (TH) herds have been described previously (17). Of the 114 isolates, 43 were from the NAB herd, 41 were from the AB herd, and 30 were from the TH herd. Isolates which had been maintained on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) deeps at 2°C were subcultured twice in Trypticase soy broth before being used. Following purification in Trypticase soy broth, the identity of each isolate was determined by using either the API 20E system (Analytab Products, Inc., Plainville, N.Y.) or the Sensititer Automated Computerized ID/MIC System (Radiometer America, Inc., Westlake, Ohio) according to the directions of the manufacturers.

Plasmid-containing *Escherichia coli* χ2556 (2.1, 2.7, 3.0,

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TABLE 1. DNA probes constructed and used in hybridization experiments

Tetracycline resistance determinant	Restriction fragment used as DNA probe	Reference
TetA	750-bp *Sma*I fragment of pSL18	24
TetB	1,275-bp *Hin*cII fragment of pRT29	24
TetC	929-bp *Bst*NI fragment of pBR322	24
TetD	3,050-bp *Hin*dIII-*Pst*I fragment of pSL106	24
TetE	2,500-bp *Cla*I-*Pvu*I fragment of pSL1504	22
TetO	1,458-bp *Hin*dIII-*Nde*I fragment of pAT121	36
TetM	850-bp *Eco*RI-*Hin*dIII fragment of pAT101	4

5.1, 5.55, and 53.7 kb), χ1792 (93 kb), χ2026 (60 kb), HB101(pRK290) (20 kb), and HB101 (pRZ102) (12 kb) (9, 21, 33) and *Agrobacterium tumefaciens* 1D1145 (180 kb) and 1D1422 (44 kb) (10) were used as reference standards to determine plasmid size. The *E. coli* strains were grown in Luria-Bertani broth (Difco Laboratories, Detroit, Mich.) at 37°C, and the *Agrobacterium tumefaciens* strains were grown at 30°C in Luria-Bertani broth.

Determination of MICs. A microdilution broth procedure performed with Mueller-Hinton supplement broth (Difco Laboratories) was used to determine the MIC of tetracycline for each isolate (13). MIC 96-well microtiter plates were incubated at 37°C for 17 h. The MIC was defined as the concentration that prevented growth.

Preparation of DNA probes. The plasmids carrying the different types of tetracycline resistance determinants are listed in Table 1. Plasmids were extracted by the method of Birnboim and Doly (3). All restriction endonucleases, including *Sma*I, *Hin*cII, *Bst*NI, *Hin*dIII, *Cla*I, *Pst*I, *Pvu*I, *Nde*I, and *Eco*RI, were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), New England BioLabs (Beverly, Mass.), or Boehringer Mannheim GmbH (Mannheim, Germany) and were used as directed by the instructions of the manufacturer. The purified restriction DNA fragments used as probes (Table 1) were labeled in vitro with [α-^{32}P]dCTP (Du Pont Co., NEN Research Products, Boston, Mass.) by using a Random Primed DNA Labeling kit (Boehringer Mannheim).

Screening isolates for plasmid DNA. The 114 strains were screened for plasmid DNA by using the method of Kado and Liu (14). Plasmid DNA was fractionated by agarose gel electrophoresis. Several plasmids having known molecular sizes, isolated from *E. coli* and *Agrobacterium tumefaciens* reference strains, were run in parallel with the plasmid DNAs from the test strains. The migration distances of the plasmid bands were measured directly from photographs of the gels. Molecular weight standard curves and size estimates for unknown plasmids from test strains were determined by performing a linear regression analysis of $\log_{10}$ molecular size versus $\log_{10}$ migration distance (27).

DNA-DNA hybridization. Plasmids were extracted by the method of Kado and Liu (14). An alkaline Southern method (31) was used to transfer fractionated plasmid DNA from agarose gels to Zeta-Probe membrane filters (Bio-Rad Laboratories, Inc., Richmond, Calif.). The filters were hybridized with the ^{32}P-labeled tetracycline resistance probes (1×10^6 cpm per filter) under stringent conditions in 0.5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate, 1 mM EDTA [pH 7.4]) containing 0.5% BLOTTO (Difco nonfat powdered milk), and 1% sodium dodecyl sulfate (SDS) for 20 h at 65°C. Zeta-Probe membranes were sandwiched between two sheets of no. 589 filter paper (Schleicher & Schuell, Keene, N.H.) and placed into hybridization bags. After hybridization, the blots were rinsed briefly in 2× SSPE and then washed successively in 2× SSPE–0.1% SDS, 0.5× SSPE–0.1% SDS, and 0.1× SSPE–0.1% SDS at 25°C for 15 min with vigorous agitation. The last wash was in 0.1× SSPE–1% SDS at 25°C for 30 min, and this was followed by rinsing in 0.1× SSPE at 25°C. The membranes were blotted dry and autoradiographed by using Kodak X-Omat AR X-ray film with intensifying screens exposed at −70°C for an appropriate length of time.

TABLE 2. Plasmid profiles of 114 gram-negative enteric bacteria isolated from swine

Herd	Reaction to tetracycline	No. of isolates	% of isolates with[a]: Large plasmids (>30 kb)	Small plasmids (<30 kb)	No plasmid
NAB[b]	Resistant	24	92	4	4
	Sensitive	19	53	11	37
AB[c]	Resistant	35	100	0	0
	Sensitive	6	50	0	50
TH[d]	Resistant	19	90	5	5
	Sensitive	11	36	9	55
Total	Resistant	78	95	3	3
	Sensitive	36	47	8	44

[a] Isolates were classified on the basis of the largest plasmid detected.
[b] The NAB herd had not been exposed to antimicrobial agents for more than 146 months.
[c] The AB herd routinely received chlortetracycline in its feed.
[d] Iowa Veterinary Medicine Research Institute herd, which received penicillin G plus streptomycin therapeutically as needed.

Total bacterial DNA extraction was performed as described by Ausubel et al. (2). Dot blot hybridization was performed by using the following procedure. A total of 34 bacterial chromosomal DNAs were heated at 100°C for 10 min and then located on Zeta-Probe membranes by using a 96-well Bio-dot microfiltration apparatus (Bio-Rad Laboratories). The membranes were dried and hybridized at 65°C overnight by using stringency conditions that were the same as the conditions used for Southern hybridization. Positive and negative controls were included in each set.

Statistical analysis. Chi-square analyses were used to compare the tetracycline resistance determinant distributions (35). A probability (P) value of 0.05 was used as the discriminator of statistical significance.

RESULTS

Plasmid profiles of enteric bacteria isolated from pigs in three herds with different histories of antimicrobial agent exposure. Gram-negative isolates from pigs in three herds with different histories of antimicrobial agent exposure were examined for the presence of plasmids and were classified according to plasmid size (Table 2). Plasmids which migrated in agarose gels slower or faster than a 30-kb marker were designated large and small plasmids, respectively (6). Tetracycline-resistant isolates, especially isolates from the AB herd, tended to have a predominance of plasmids whose molecular sizes were greater than 30 kb. A higher proportion of tetracycline-sensitive strains than tetracycline-resistant strains were plasmid free or harbored small plasmids (<30 kb). About 50% of the tetracycline-sensitive strains isolated from the AB herd and the NAB herd and 36% of the strains isolated from the TH herd contained large plasmids (>30

TABLE 3. Distribution of tetracycline resistance determinants on plasmids for isolates from three swine herds with different histories of antimicrobial agent exposure

Herd	No. of pigs	No. of isolates	% of isolates carrying the following tetracycline resistance determinants on plasmids:						
			TetA	TetB	TetC	TetD	TetA and TetB	TetA and TetC	None[a]
NAB[b]	19	24	4	17	29	0	0	0	50
AB[c]	23	35	0	51	6	0	3	11	29
TH[d]	14	19	0	26	0	0	0	11	63
Total	56	78	1	35	12	0	1	8	43

[a] The strains did not hybridize with TetA, TetB, TetC, and TetD tetracycline resistance determinants.
[b] The NAB herd had not been exposed to antimicrobial agents for more than 146 months.
[c] The AB herd routinely received chlortetracycline in its feed.
[d] Iowa Veterinary Medicine Research Institute herd, which received penicillin G plus streptomycin therapeutically as needed.

kb). These results suggest that factors other than the subtherapeutic and/or therapeutic use of antimicrobial agents select for large plasmids.

Distribution of tetracycline resistance determinants in isolates from three swine herds with different histories of antimicrobial agent exposure. The plasmids of 114 isolates were extracted by the method of Kado and Liu (14), electrophoresed on agarose gels, and then transferred to membranes; this was followed by hybridization with TetA, TetB, TetC, and TetD tetracycline resistance probes. The DNA-DNA hybridization results and the distribution of the tetracycline resistance determinants on plasmids in isolates from the three herds are shown in Table 3. None of the tetracycline-sensitive isolates ($n = 36$) (MIC, ≤ 4 μg/ml) hybridized with the TetA, TetB, TetC, and TetD probes. Approximately 44% (34 of 78) of the plasmid DNAs isolated from tetracycline-resistant isolates did not hybridize with the TetA, TetB, TetC, and TetD DNA probes. None of the isolates hybridized with the TetD determinant probe. Of the 44 tetracycline-resistant isolates which hybridized with the probes, 35% carried only the TetB determinant, and 12 and 1% of the strains carried only the TetC determinant and only the TetA determinant, respectively. A total of 1% carried both the TetA and TetB determinants, while 8% of the strains carried both the TetA and TetC determinants.

The distribution of the tetracycline resistance determinants on plasmids among isolates from the NAB herd was different from the distribution on plasmids among isolates from the AB herd ($P < 0.05$). TetC was the most prevalent determinant in tetracycline-resistant isolates from the NAB herd, followed by TetB and TetA. A total of 50% of the isolates from the NAB herd did not carry any TetA, TetB, TetC, or TetD determinant on their plasmids. TetB was the predominant determinant (51%) on plasmids of tetracycline-resistant isolates from the AB herd, followed by TetC (6%). Several tetracycline-resistant isolates from the AB herd contained both TetA and TetB determinants (3%) or both TetA and TetC determinants (11%). A total of 29% of the isolates did not hybridize with the probes. The TetB determinant (26%) was the most common determinant observed for the plasmids of tetracycline-resistant isolates from the TH herd; however, 63% of the isolates did not hybridize with any of the five probes. A total of 11% of the isolates from the TH herd carried both TetA and TetC determinants.

The 34 isolates whose plasmids did not hybridize with the TetA, TetB, TetC, and TetD probes were reevaluated to determine whether they carried tetracycline resistance determinants on chromosomes. The chromosomal DNAs of these 34 isolates were extracted and fixed on membranes by using a dot blot apparatus; this was followed by hybridization with TetA, TetB, TetC, TetD, TetE, TetM, and TetO probes. The hybridization results are shown in Table 4. All of the isolates exhibited homology with probes for the TetB or TetE determinant. Approximately 15% (5 of 34) of the isolates carried the TetB determinant on chromosomal DNA, and 9% (3 of 34) carried the TetE determinant. A large proportion of the isolates (76%) carried both TetB and TetE determinants on chromosomes. No isolate carried TetA, TetC, or TetD tetracycline resistance genes on its chromosome. The NAB herd isolates carried more TetE determinants than TetB determinants on their chromosomes, while the AB herd and TH herd isolates carried more TetB determinants than TetE determinants on their chromosomes.

No signal was observed when plasmid DNAs from these 34 isolates were extracted and hybridized with TetE, TetM, and TetO probes. Our results show that these 34 isolates

TABLE 4. Distribution of tetracycline resistance determinants on chromosomes for 34 isolates which did not carry tetracycline resistance determinants on plasmids

Herd	No. of pigs	No. of isolates	% of isolates carrying the following tetracycline resistance determinants on chromosomes:							
			TetA	TetB	TetC	TetD	TetE	TetB and TetE	TetM	TetO
NAB[a]	11	12	0	17	0	0	25	58	0	0
AB[b]	9	10	0	20	0	0	0	80	0	0
TH[c]	9	12	0	8	0	0	0	92	0	0
Total	29	34	0	15	0	0	9	76	0	0

[a] See Table 2, footnote *b*.
[b] See Table 2, footnote *c*.
[c] See Table 2, footnote *d*.

TABLE 5. Presence of TetE tetracycline resistance determinants

Species	No. of isolates tested	No. of isolates with TetE	
		TetE	TetB and TetE
E. coli	31	3	23
S. enteritidis	1	0	1
Unidentified	2	0	2
Total	34	3	26

carried TetE determinants only on chromosomes, not on plasmids.

The identities of the 34 isolates which carried TetE determinants are shown in Table 5. *E. coli* strains with TetE determinants accounted for 90% (26 of 29) of the isolates. In addition to *E. coli*, isolates identified as *Salmonella enteritidis* also carried the TetE determinant.

Association of tetracycline resistance levels with strains bearing different tetracycline resistance determinants. Strains carrying various tetracycline resistance determinants had different MICs (Table 6). A correlation between MICs and tetracycline resistance determinants on plasmids or on chromosomal DNAs was found to be significant ($P < 0.05$), as determined by chi-square analyses. Bacteria carrying the TetC determinant on plasmids had lower MICs (8 to 64 μg of tetracycline per ml) than bacteria carrying TetA and B determinants (128 μg of tetracycline per ml) ($P < 0.05$).

Of the isolates with the TetB determinant on plasmids and chromosomes, 93 and 100%, respectively, had an MIC of 128 μg of tetracycline per ml. In contrast, most isolates (89%) with the TetC determinant had MICs of less than 64 μg of tetracycline per ml. Strains carrying two different tetracycline resistance determinants (e.g., TetA and TetB or TetB and TetE) had MICs of 128 μg of tetracycline per ml and did not exhibit increased tetracycline resistance. Bacteria carrying TetA and TetB or TetB and TetE determinants had MICs of 128 μg of tetracycline per ml. A total of 67% of the strains bearing TetA and TetC determinants had MICs of 128 μg of tetracycline per ml.

In our study, only one isolate carried TetA and one isolate carried TetA and TetB determinants on their plasmids; therefore, a larger sample size might be needed in future studies to confirm this result.

TABLE 6. Association between tetracycline MICs and the presence of tetracycline resistance determinants in bacterial strains from three swine herds

Location	Tetracycline resistance determinant(s)	No. of isolates	% of isolates inhibited by tetracycline at a concn of:		
			≤4 μg/ml	8-64 μg/ml	≥128 μg/ml
Plasmid	TetA	1	0	0	100
	TetB	27	0	7	93
	TetC	9	0	89	11
	TetD	0	0	0	0
	TetA and TetB	1	0	0	100
	TetA and TetC	6	0	33	67
Chromosome	TetB	5	0	0	100
	TetE	3	0	33	66
	TetB and TetE	26	0	0	100

DISCUSSION

This is the first time that tetracycline resistance DNA probes have been used to detect the presence of a tetracycline resistance gene on plasmids or chromosomes in enteric bacteria from three swine herds with different histories of antibiotic use. Since the herds had defined histories of antimicrobial agent use, they provided a unique opportunity to study the molecular divergence of antibiotic resistance determinants. The DNA hybridization results demonstrated the presence of four tetracycline resistance determinants (TetA, TetB, TetC, and TetE) in enteric bacteria isolated from these three swine herds. None of the tetracycline-sensitive isolates hybridized with the tetracycline resistance DNA probes in this study. These results support the suitability of using DNA probes for detecting tetracycline resistance genes (19, 37).

The TetB tetracycline resistance determinant has been reported to be the most common tetracycline resistance determinant in members of the *Enterobacteriaceae* (24). We also found that the TetB determinant was the predominant type of tetracycline resistance determinant among the 78 tetracycline-resistant strains isolated from pigs. TetB was the predominant type of tetracycline resistance determinant in isolates from pigs exposed to antimicrobial agents (the AB and TH herds), whereas TetC and TetE were the most common tetracycline resistance determinants on plasmids and chromosomes, respectively, for isolates from pigs in a herd not exposed to antimicrobial agents for more than 146 months (the NAB herd). A lower proportion of isolates from the AB herd than from the NAB and TH herds did not carry tetracycline resistance determinants on plasmids. In contrast, a higher proportion of strains from the AB herd than from the other two herds carried the TetB determinant (Tables 3 and 4). These results may reflect the differences in the histories of antimicrobial agent exposure of the three herds.

The determinants were observed in both lactose-negative and lactose-positive fecal isolates in our results. Marshall et al. (24) reported that the TetB determinant is found most frequently in lactose-fermenting coliform bacteria isolated form animal fecal samples. However, we found that the TetB determinant occurred more frequently in the lactose-negative than in the lactose-positive *E. coli* strains which we studied (data not shown). Marshall et al. (24) found that the TetA determinant occurred more frequently than the TetC determinant did. In contrast to the results of Marshall et al. (24), we observed only one isolate that carried the TetA determinant. However, seven isolates carried the TetA determinant along with the TetB or TetC determinant. Mendez et al. (26) found the TetC determinant on plasmids isolated from *Salmonella* species, and they suggested that the TetC determinant may be more common on plasmids in *Salmonella* species. The *Salmonella* strain tested in our study did not carry the TetC determinant on a plasmid but did carry the TetE determinant on the chromosome. Our observation of a *Salmonella* species with the TetE determinant has not been reported by any other researchers.

The TetD determinant is rarely found in lactose-fermenting coliform bacteria (24), but it is common in fish pathogens (1). The TetM and TetO genes diverged from a common ancestor (36), but differ in their distributions (both of them are present in some gram-negative bacteria) (28, 32, 36). However, TetD, TetM, and TetO resistance determinants were not found in the isolates tested.

Several strains carried more than one type of tetracycline

resistance gene on plasmids (e.g., TetA and TetB, or TetA and TetC); however, each type was carried on a separate plasmid. Plasmids larger than 30 kb were detected in both tetracycline-resistant (95%) and tetracycline-sensitive (47%) gram-negative fecal isolates from pigs in three herds with different histories of antimicrobial agent exposure (Table 2). The large plasmids in the tetracycline-sensitive isolates may be associated with resistance to antimicrobial agents other than the tetracyclines or with unknown functions, since the majority of the isolates used in this study exhibited multiple antibiotic resistance (data not shown).

The TetE determinant has been found to be rare in *E. coli* lactose-fermenting fecal isolates (1 of 215 isolates tested) (22), and it has not been reported in any species other than *Aeromonas hydrophila* (8). Our study is the first to show that a rather sizable proportion of wild-type tetracycline-resistant *E. coli* strains (26 of 34) carried the TetE determinant on their chromosomes. In addition, one *S. enteritidis* strain also had acquired the TetB and TetE tetracycline resistance determinants (Table 5). These results suggest that DNA probes may be useful in evaluating the passage of resistance determinants between groups of animals.

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