Environ. Sci. Technol. **2010,** *44,* 6933–6939

Abundance and Diversity of Tetracycline Resistance Genes in Soils Adjacent to Representative Swine Feedlots in China

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Received March 11, 2010. Revised manuscript received July 28, 2010. Accepted August 2, 2010.

Tetracyclines are commonly used antibiotics in the swine industry for disease treatment and growth promotion. Tetracycline resistance was determined in soils sampled from farmlands in the vicinity of nine swine farms located in three cities in China. Totally, 15 tetracycline resistance (*tet*) genes were commonly detected in soil samples, including seven efflux pump genes (*tet*A, *tet*C, *tet*E, *tet*G, *tet*K, *tet*L, *tet*A/P), seven ribosomal protection proteins (RPPs) genes (*tet*M, *tet*O, *tet*Q, *tet*S, *tet*T, *tet*W, *tet*B/P), and one enzymatic modification gene (*tet*X). The quantitative real-time PCR was further used to quantify five RPPs genes (*tet*M, *tet*O, *tet*Q, *tet*W, *tet*T) and 16S rRNA gene abundances. The concentrations of total tetracyclines (5 typical tetracyclines and 10 of their degradation products) in these soils were measured using liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) and were found to range from 5.4 to 377.8 $\mu g \cdot kg^{-1}$ dry soil. Bivariate correlation analysis confirmed that absolute *tet* gene copies (sum of *tet*M, *tet*O, *tet*Q, *tet*W genes) were strongly correlated with the concentrations of tetracycline residues ($r^2 = 0.45$, $P < 0.05$), ambient bacterial 16S-rRNA gene copies in each soil sample ($r^2 = 0.65$, $P < 0.01$), and organic matter in soil ($r^2 = 0.46$, $P < 0.05$), respectively. Finally, the phylogenetic analysis on *tet*M combined with culture-independent molecular techniques revealed at least five genotypes of *tet*M in nine soil samples.

Introduction

In concentrated animal feeding operations, antibiotics are routinely used for disease control and growth promotion (*1*). However, when used in this manner, antibiotics can select for resistant bacteria in the gastrointestinal tracts of production animals, providing a potential reservoir for dissemination of resistant bacteria into the environment (*2*). Bacteria have been shown to readily share genetic information by horizontal gene transfer mechanism driven by mobile genetic elements (*3*), permitting the transfer of resistance genes from fecal microorganisms to indigenous environmental bacteria. Furthermore, many antibiotics are poorly absorbed in the animal gut and subsequently could be excreted into the environment by feces (*1*) where they tend to persist and accumulate in soils after repeated manure application (*4*). Residual antibiotics may exert selection pressure on environmental microorganisms, contributing to the spread of antibiotic resistant microorganisms and the resistance genes they carry (*5*). Growing evidence exists that antibiotics used in the animal industry may transfer antibiotic resistance genes into the environment through a variety of pathways connected with common waste disposal practices, such as land application of manures (*1, 6*). However, it is difficult to demonstrate a direct link between agricultural usage of antibiotics and increased levels of resistance in pathogens (*7*), the potential consequences are serious, and the World Health Organization has recommended that antimicrobials be phased out as animal growth promoters (*8*). In China, more than 8000 tons of antibiotics are used as feed additives each year (*9*), but there is no regulation on antibiotics used as feed additives for growth promotion at present (*9*) or legislation in China for the limits of antibiotics in the environment.

Tetracyclines (TCs) are broad-spectrum antibiotics, which are extensively used in clinical treatment and livestock industry, particularly in the swine industries (*10, 11*). In China, the annual tetracycline usage was about 9413 tons in 1999 (*12*); in 2003, the annual oxytetracycline production in China was about 10,000 tons (65% of world total) (*13*). The majority of TCs are excreted into the environment via various pathways (e.g., agricultural runoff), and there is potential for resistance selection among pathogens and nonpathogens (*8, 14*). To date, more than 40 classes of tetracycline resistance (*tet*) genes have been described in various environments (*15, 16*), which encode three main resistance mechanisms (efflux pump proteins, ribosomal protection proteins (RPPs), and inactivating enzymes) (*16*). In China, tetracycline-resistant bacteria (e.g., *Escherichia coli*) and *tet* genes have been found in different water environments (*12, 13*). However, little is known about the concentrations of tetracycline residues and the levels of *tet* genes in soil environments in China.

Previous studies investigated various *tet* genes in environments surrounding swine feedlots (*2, 10, 11, 17*), swine manures, or piggery manured soils (*18*−*20*), indicating the ubiquitous occurrence of *tet* genes in environments influenced by swine farms. The quantitative real-time PCR (qRT-PCR) assay has been frequently employed to study the resistance levels (*11, 21*−*23*). A number of studies reported that *tet* gene concentrations were significantly correlated with the levels of tetracycline residues in the environment (*21, 24, 25*); however, only parent TCs were investigated as indicators for TCs levels thereby omitting any consideration of degradation products. It is unlikely that this correlation is indicative of a true relationship, because the presence of tetracycline degradation products would theoretically select for maintenance and transfer of tetracycline resistance as they have potency on tetracycline sensitive bacteria (*26*). Among the *tet* genes, *tet*M is known to have the widest host range of any *tet* genes (*16*) since it is often associated with mobile genetic elements which enhance its transferability from one bacteria to another (*27*). Several studies have reported a variety of genotypes of the *tet*M from natural

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10.1021/es1007802
Published on Web 08/13/2010

environments (*28, 29*) or resistance isolates (*10, 30*), and various genotypes of the *tet*M are known to occur in human and animal pathogens (*29*).

To evaluate the effects of swine production facilities on the levels of resistance to tetracyclines in surrounding soil environments and to fill the knowledge gaps at the national level, a survey was designed to investigate the antibiotics residues and determine the occurrence and quantity of resistance genes in soil environments adjacent to swine facilities in China. In this study, soil samples were collected from farmlands surrounding nine swine feedlots from three cities in China, and PCR was used to determine the occurrence of *tet* genes typically present in soil samples. Seven efflux pump genes (*tet*A, *tet*C, *tet*E, *tet*G, *tet*K, *tet*L, *tet*A/P), seven ribosomal protection proteins genes (*tet*M, *tet*O, *tet*Q, *tet*S, *tef*T, *tet*W, *tet*B/P), and one enzymatic modification gene (*tet*X) were selected, since these genes have been reported to frequently occur in environments surrounding swine feedlots (*10, 11, 17, 31*), and they reflect the three main resistance mechanisms encoded by *tet* genes. Furthermore, the qRT-PCR was used to quantify five RPPs genes *tet*M, *tet*O, *tet*Q, *tet*W, and *tef*T, which were chosen because some studies found greater relative abundance of RPPs genes than that of *tet* efflux pump genes in swine feedlots (*8, 11*), they have established relationship with bacteria of public health concern (*16, 32*), and the real-time PCR method targeting these genes has been established during our preliminary tests. To investigate the levels of tetracycline residues in soil samples, liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) methods were used to determine the concentrations of five typical tetracyclines and ten of their degradation products. Finally, the phylogenetic analysis was performed to investigate the diversity of *tet*M genotypes; culture-independent molecular techniques were used in phylogenetic analysis because the conventional culture-dependent methods are limited in scope by only considering culturable bacteria (*28*).

Experimental Section

Soil Sampling and Processing. Soil samples were collected from farmlands adjacent to nine swine production facilities located in three cities—Beijing, Tianjin, and Jiaxing (in Zhejiang Province) (Figure S1) between November 2008 and June 2009. In Beijing, samples were taken around two swine feedlots in Changping District (define as BJ1 and BJ2, respectively), and one feedlot in Shunyi District (define as BJ3); in Tianjin, samples were from Beichen District (TJ1 and TJ2) and Dagang District (TJ3); in Jiaxing, samples were from Nanhu District (JX1 and JX2) and Tongxiang (JX3). For each site, three subsamples were collected from the top 0 to 10 cm of the surface soil and were mixed to form one composite sample. All samples were passed through a 2.0 mm sieve, and subsamples were stored at −80 °C for DNA extraction. Soil pH was determined with a soil to water ratio of 1:10. Soil organic matter was determined by the $K_2Cr_2O_7$ oxidation method.

DNA Extraction. Total DNA was extracted from 0.5 g of soil using a FastDNA SPIN kit for Soil (Bio 101 Inc., Vista, CA), following the manufacturer's instructions. The concentration and quality of the extracted DNA was determined by spectrophotometer analysis (NanoDrop ND-1000, NanoDrop Technologies, Willmington, DE) and agarose gel electrophoresis.

PCR Assays for Detection of tet Genes. All PCR assays were conducted in a 25 μL volume reaction, using a Peltier Thermal Cycler (Bio-Rad, Hercules, CA). The PCR mixture consisted of 2.5 μL 10× Ex Taq buffer (Mg^{2+} Plus); 2 μL dNTPs (2 mM each); 0.2 μM of each primer; 1.25 U of Ex Taq DNA Polymerase; and 0.5 μL of template. Primers targeting 15 different *tet* genes, *tet*A, *tet*C, *tet*E, *tet*G, *tet*K, *tet*L, *tet*A/P, *tet*B/P, *tet*M, *tet*O, *tet*Q, *tet*S, *tef*T, *tet*W and *tet*X were described previously (*30, 33*), and their sequences are listed in Table S1 (Supporting Information). The temperature program was initially denaturated at 94 °C for 4 min, followed by 35 cycles of 45 s at 94 °C, 45 s at different annealing temperatures (Table S1), and 72 °C for 1 min, with a final extension step for 6 min at 72 °C. PCR products were analyzed by electrophoresis on a 1.5% agarose gel in 1× TAE buffer. To ensure reproducibility, duplicate PCR reactions were performed for each sample. Sterile water was used as the negative control in every run.

After PCR amplification, gel slices of an agarose gel containing the PCR products were excised and purified using Agarose Gel DNA Purification Kit (TaKaRa). The purified PCR products were ligated into p-GEM T easy vector (Promega, Madison, WI) and then cloned into *Escherichia coli* JM109 (TaKaRa). Clones containing *tet* gene inserts were picked and sequenced. If the gene inserts were verified as the object resistance genes using the BLAST alignment tool (http://www.ncbi.nlm.nih.gov/blast/), clones that had the right *tet* gene inserts were chosen as the positive control for qualitative PCR as well as the standards for the real-time PCR. Plasmids carrying target genes were extracted with Plasmid Kit (TaKaRa).

qRT-PCR Methods. Five target genes (*tet*M, *tet*O, *tet*Q, *tet*W, and *tef*T) and 16S rRNA were quantified based on the fluorescent dye SYBR-Green I. Plasmids carrying target genes were used to generate calibration curves, and their concentrations were measured with a spectrophotometer (Nanodrop). As the lengths of the vector and target gene inserts were known, the gene copy numbers were calculated directly from extracted plasmid DNA concentration (*34*). 10-fold serial dilutions of a known copy number of the plasmid DNA were generated to produce the standard curve. The PCR efficiencies (88.8−119.5%) were examined to test for inhibition. R^2 values were more than 0.990 for all calibration curves. Data analysis was carried out with iCycler software (version 1.0.1384.0 CR).

The 16S rRNA and *tet* gene copy numbers were determined in triplicate using an iCycler IQ5 Thermocycler (Bio-Rad, Hercules, CA). Probe/primer sets for 16S rRNA genes were adapted from Suzuki and colleagues (*35*). The qPCR primer sets for *tet* genes were the same as those used in qualitative PCR. Each reaction was performed in a 25 mL volume containing 12.5 μL of SYBR Premix Ex Taq (TaKaRa), 0.2 μM of each primer, and 2 μL of template DNA which was diluted 10-fold. The PCR protocol was as the following program: 1 min at 95 °C, followed by 40 cycles of 10 s at 94 °C, 45 s at the annealing temperatures. The temperature at which the fluorescence was read during each cycle was varied according to different target genes (79 °C for *tef*T, 80 °C for *tet*M, 81 °C for *tet*O, 82 °C for *tet*Q, and 83 °C for *tet*W). Product specificity was confirmed by melting curve analysis (55−95 °C, 0.5 °C per read, 30 s hold) and visualization in agarose gels.

Tetracyclines Detection. The procedures for extraction of tetracyclines and their degradation products in soils followed those described by Kim and Carlson (*36*) with minor modifications. LC-MS/MS was used to separate and detect the tetracyclines, following methods described by Jia (*14*). Five target tetracyclines, including tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), doxycycline (DXC), and methacycline (MTC), and ten degradation products, including 4-epitetracycline (ETC), anhydrotetracycline (ATC), 4-epianhydrotetracycline (EATC), 4-epioxytetracycline (EOTC), α-apo-oxytetracycline (α-apo-OTC), β-apo-oxytetracycline (β-apo-OTC), isochlortetracycline (ICTC), 4-epichlortetracycline (ECTC), anhydrochlortetracycline (ACTC), and 4-epianhydrochlortetracycline (EACTC), were analyzed in this study.

Cloning and Phylogenetic Analysis of tetM Genes. The clone libraries of *tet*M amplicons obtained from all nine soil

samples were constructed to analyze the sequence diversity. *Tet*M gene fragments were cloned as described above. Between 20 and 30 representative clones from each library bearing inserts of the correct size (171 bp) were randomly selected for sequencing. Online similarity searching was performed using the BLAST tool. Sequences were subjected to homology analysis using the software DNAMAN version 6.0.3.48 (Lynnon Biosoft, USA). For sequence types that exhibited more than 98% identity to each other, only one representative was considered for construction of trees. The GenBank sequences most similar to clone sequences in this study and reference sequences for defining clusters were included in phylogenetic tree construction. Nucleotide sequences of *tet*M were aligned using the multiple sequence alignment program CLUSTAL W (*37*). The neighbor-joining trees were constructed using MEGA version 4.1 (http://megasoftware.net/). Bootstrap analysis with 1000 replicates was used to evaluate the significance of the nodes.

The sequences of *tet*M obtained in this study have been deposited in GenBank under Accession No. GU373619 to GU373623, GU474957 to GU474981.

Data Analysis. Copy numbers were log-transformed as needed to normalize the distributions prior to statistical analysis. All statistical analyses were performed using SPSS version 13.0, and one-way analysis of variance (ANOVA) was used to assess the homogeneity of variance with significance levels of 5% ($P < 0.05$). Pearson's bivariate correlation analysis was used to assess trends among *tet* genes and other parameters monitored.

Results and Discussion

Occurrence of tet Genes. All 15 *tet* genes were detected in soil samples adjacent to swine feedlots, and each sample contained several different *tet* genes (Table S2). The soil samples from different sites showed different diversities of resistance genes. Samples from TJ1 and TJ2 contained 12 *tet* gene patterns, while samples from JX3 only contained seven *tet* gene patterns. Two efflux pump genes (*tet*A, *tet*C), four RPPs genes (*tet*M, *tet*O, *tet*Q, *tet*W), and one enzymatic modification gene (*tet*X) were the most frequently detected determinants in soil samples. Among them, four RPPs genes (*tet*M, *tet*O, *tet*Q, *tet*W) have been detected in all samples, while *tet*E and *tet*S genes were only detected in one sample (TJ1).

The different diversities of resistance genes in soils from different sites possibly reflect regional differences in gene diversity. Schmitt et al. found that the diversity of resistance genes from German agricultural field was considerably lower than that of Swiss pasture (*18*). All 15 *tet* genes detected in the present study were also reported previously in swine lagoons (*2, 10, 11, 17*), in swine manures, or piggery manured soils (*18*−*20*). These results were consistent with the hypothesis that the seepage from waste lagoons and/or land application of manure waste from swine production facilities nearby contributes to the spread of these *tet* genes in surrounding soil environments. However, it is known that resistance could occur in pristine soil environments even without any record of antibiotic contamination (*5, 38*), so it is not possible to conclude whether this diversity is caused by the spread of resistance after 50 years of tetracycline use or is due to the natural background in soil resistance genes (*18*). But the evidence that a high diversity of resistance genes were detected in soil in the present study still showed that soil plays an important role as resistance gene reservoir (*39*).

The most frequently detected seven *tet* genes in soil samples covered all the three main resistance mechanisms, which showed that the detection frequency of *tet* genes had no preference for the resistance mechanism (*34*). Four RPPs genes (*tet*M, *tet*O, *tet*Q, and *tet*W) have been detected in all samples, indicating their prevalence in soils surrounding swine feedlots. Similar results were also found in swine lagoons where *tet*M, *tet*O, *tet*Q, and *tet*W were dominant in the total communities (*10*). The prevalence of these four RPP genes in the present study is not surprising, since these genes were found to be predominant in the gastrointestinal tracts of pigs and steers (*30*), and the increasing frequencies of these genes in the vicinity of pig farms are consistent with the hypothesis that this occurrence could be the result of gene flow from the animals (*10*). The above aspects are in favor of using these four RPPs genes as indicators to assess the tetracycline resistance in soils adjacent to swine feedlots. Therefore, *tet*M, *tet*O, *tet*Q, and *tet*W genes were further quantified using qRT-PCR.

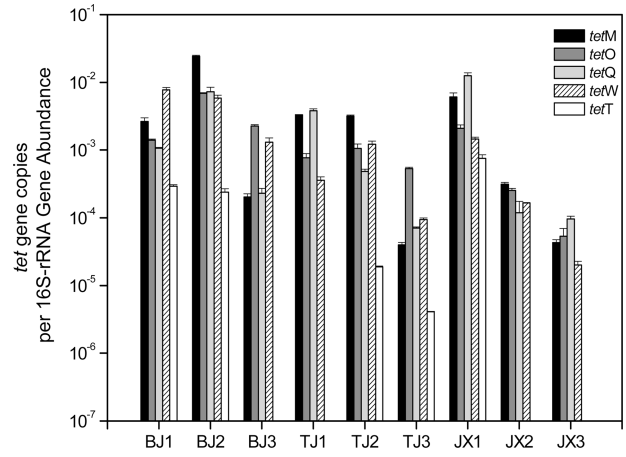

FIGURE 1. Detected levels of five tetracycline resistance genes (*tet*M, *tet*O, *tet*Q, *tet*W, and *tet*T) normalized to copies of ambient 16S rRNA gene present in soil samples from different sites. Error bars indicate the standard deviation of three replicates.

The limited distribution of some genes is possibly associated with their limited host range, such as *tet*E, since it is associated with large plasmids, which are neither mobile nor conjugative (*33*). That reduces its opportunities for transfer to other species (*32*). However, the limited distribution of *tet*S is not expected since it can be found in conjugative plasmids although they are not self-mobile (*32*).

In this survey only a limited number of resistance genes in soils were investigated, however, a variety of ARGs encoding different antibiotic resistance could be present in soil environments in the vicinity of swine feedlots where different classes of antibiotics (e.g., macrolides, lincosamides) are frequently used in addition to TCs (*1*). So there is a need for further study to explore more diverse ARGs in soils. In addition, it is noteworthy that many ARGs have been found frequently to be part of multiple antibiotic resistant gene cassettes which can cause multiple antibiotic resistance in bacteria, meaning that when one antibiotic was used, it may coselect for resistance to other different antibiotics simultaneously (*3*). So a comprehensive study on multiple antibiotic resistance in soils surrounding swine feedlots is important.

Quantification of tet Genes in Soils around Swine Feedlots. Absolute gene copy numbers of five *tet* genes (*tet*M, *tet*O, *tet*Q, *tet*W, and *tet*T) in soil samples are presented in Table S3. To minimize variance caused by different extraction and analytical efficiencies, and differences in background bacterial abundances, the absolute number of all resistance genes was normalized to that of ambient 16S rRNA genes (Figure 1 and Table S4). The same general trends in gene abundance were seen in the normalized data (Table S4) relative to absolute data (Table S3), since the total number of 16S rRNA gene copies was found to be relatively consistent among different sites at 10^{10} copies per gram dry soil, except for JX3 (10^9 copies per gram dry soil) (Table S5). The relative

TABLE 1. Quantification of Tetracyclines in Soil Samples, Normalized to the Dry Weight of the Soil[a]

	swine feedlots (μg/kg)								
compound	BJ1	BJ2	BJ3	TJ1	TJ2	TJ3	JX1	JX2	JX3
tetracycline (TC)	10.57 (1.64)	95.28 (55.50)	13.66 (7.52)	1.32 (0.12)	2.74 (0.69)	6.12 (3.07)	3.10 (0.32)	1.33 (0.19)	1.40 (0.31)
4-epitetracycline (ETC)	11.08 (4.05)	135.58 (78.84)	14.39 (2.72)	0.78 (0.17)	2.43 (0.82)	2.59 (0.59)	2.79 (0.35)	0.66 (0.21)	0.62 (0.12)
anhydrotetracycline (ATC)	7.00 (3.29)	4.51 (2.52)	9.92 (1.04)	1.07 (0.56)	4.46 (1.60)	4.03 (0.60)	1.57 (0.60)	0.70 (0.26)	1.28 (0.54)
4-epianhydrotetracycline (EATC)	7.50 (3.60)	5.80 (2.84)	11.63 (0.35)	4.12 (1.04)	3.70 (1.81)	3.61 (0.62)	0.87 (0.17)	0.67 (0.13)	0.60 (0.17)
oxytetracycline (OTC)	13.12 (2.90)	20.36 (14.06)	33.21 (12.91)	ND	0.41 (0.07)	4.51 (1.09)	3.24 (0.73)	0.29 (0.06)	ND
4-epioxytetracycline (EOTC)	7.76 (3.04)	22.17 (16.81)	29.87 (16.18)	ND	ND	ND	2.46 (0.28)	ND	ND
α-apo-oxytetracycline (α-apo-OTC)	0.07 (0.02)	0.06 (0.06)	0.05 (0.02)	ND	0.08 (0.04)	0.09 (0.04)	0.09 (0.04)	0.10 (0.06)	ND
β-apo-oxytetracycline (β-apo-OTC)	0.16 (0.05)	0.09 (0.02)	ND	0.33 (0.14)	0.15 (0.05)	ND	0.13 (0.07)	0.13 (0.11)	0.08 (0.03)
chlortetracycline (CTC)	33.20 (9.39)	32.23 (18.67)	19.47 (6.43)	ND	0.69 (0.48)	0.83 (0.17)	7.77 (0.59)	1.24 (0.25)	0.36 (0.12)
4-epichlortetracycline (ECTC)	69.79 (22.23)	58.26 (33.72)	37.61 (16.84)	ND	ND	2.50 (0.80)	21.81 (6.70)	2.40 (1.26)	ND
anhydrochlortetracycline (ACTC)	0.41 (0.09)	0.73 (0.52)	0.42 (0.34)	0.60 (0.43)	0.51 (0.26)	0.89 (0.06)	0.59 (0.39)	0.15 (0.03)	0.30 (0.19)
4-epianhydrochlortetracycline (EACTC)	0.73 (0.30)	1.09 (0.24)	0.46 (0.10)	1.27 (0.83)	1.03 (0.26)	1.25 (0.35)	0.85 (0.70)	0.51 (0.33)	0.39 (0.16)
isochlortetracycline (ICTC)	1.98 (0.17)	1.42 (0.43)	0.77 (0.33)	0.21 (0.07)	ND	ND	0.85 (0.08)	0.28 (0.10)	ND
methacycline (MTC)	ND	ND	0.17 (0.04)	ND	0.10 (0.05)	ND	0.22 (0.08)	0.12 (0.04)	0.10 (0.03)
doxycycline (DXC)	0.17 (0.11)	0.18 (0.07)	0.26 (0.10)	0.19 (0.08)	0.16 (0.04)	ND	5.01 (0.13)	0.54 (0.18)	0.23 (0.06)
sum of tetracyclines	163.55 (50.88)	377.77 (224.30)	171.88 (64.91)	9.90 (3.43)	16.45 (6.16)	26.41 (7.40)	51.35 (11.25)	9.12 (3.22)	5.36 (1.74)

[a] The data represent the average of three independent sample concentrations. (Standard errors of the mean are presented in brackets.)

abundances (target gene/16S rRNA genes) of *tet*M, *tet*O, *tet*Q, and *tet*W in soil showed significant variation over sampling sites, ranging from 10^{-5} to 10^{-2}. In summary, the total absolute and relative abundances of these four *tet* genes (Tables S3 and S4) on average in different soil samples showed significant variation, in the range of 1.46×10^6 to 2.62×10^9 (about 1794 times difference) and in the range of 2.12×10^{-4} to 4.41×10^{-2} (about 208 times difference), respectively. However, *tef*T genes were lower in relative abundances (from 10^{-6} to 10^{-4}) than the other four genes at all sites where *tef*T genes were detected.

To date, four RPPs genes (*tet*M, *tet*O, *tet*Q, and *tet*W) observed in different environments showed very different relative abundances. In our results, the relative abundances of these four RPPs genes ranged from 10^{-5} to 10^{-2}, comparable to the results obtained from waste lagoons at cattle feedlots, ranging from 10^{-6} to 10^{-2} (*24*); in swine lagoons and groundwater samples, generally higher relative abundances of these four genes were presented with values in the range of 10^{-4} to 10^{-1} (*11*); however, samples from river sediments contained much lower concentrations of *tet*O and *tet*W ranging from 10^{-8} to 10^{-7} (*22*). In theory, prevalent resistance genes in soils near swine feedlots should reflect the gastrointestinal microbes of the involved animals and the tendency of different genes (and/or their hosts) to survive after release into the environment (*24*). The prevalence of these four RPPs genes could be attributed to their predominance in the gastrointestinal tracts of pigs, as previously mentioned (*30*), and their elevated possibilities of transfer from one bacteria to another because of their close relationship with mobile genetic elements such as plasmids, conjugative transposons, integrons, and consequently their wide host range (*16, 32*).

Although *tef*T was not always detected in these samples in the present study (Table S2), it has been reported to be ubiquitous in soil and pig slurries (*18*). Therefore *tef*T was also quantified simultaneously for comparison purposes. In the present study, *tef*T genes were lower in relative abundances than the other four RPPs genes (*tet*M, *tet*O, *tet*Q, and *tet*W). It could be attributed to the limited transferability of *tef*T genes compared with the other four genes or the shorter migration of *tef*T gene carriers from manure to soil. The *tef*T gene has been found in the pathogen *Streptococcus pyogenes* (*18*), but very few studies reported its potential host range (*16*). The higher concentrations of *tet*M, *tet*O, *tet*Q, and *tet*W than that of *tef*T in samples at all the sites also suggested that the risk for dissemination of *tet*M, *tet*O, *tet*Q, and *tet*W in environments is much higher than that for *tef*T.

Quantification of Tetracyclines in Soils. Table 1 summarizes the concentrations of five typical tetracyclines and ten of their degradation products in soils. From these findings, it was concluded that a degradation of tetracyclines occurred. TC (average concentration range 1.3−95.3 $\mu g \cdot kg^{-1}$ dry soil) and its three degradation products ETC (0.6−135.6 $\mu g \cdot kg^{-1}$ dry soil), ATC (0.7−9.9 $\mu g \cdot kg^{-1}$ dry soil), and EATC (0.6−11.6 $\mu g \cdot kg^{-1}$ dry soil) and two degradation products ACTC (0.1−0.9 $\mu g \cdot kg^{-1}$ dry soil) and EACTC (0.4−1.3 $\mu g \cdot kg^{-1}$ dry soil) from CTC were observed in all soils. Among them, the average concentration of ETC observed in the BJ2 sample was the highest, up to 135.6 $\mu g \cdot kg^{-1}$ dry soil. Totally, the highest concentration of the total tetracyclines from BJ2 soil (377.8 ± 224.3 $\mu g \cdot kg^{-1}$ dry soil) were about 2 orders of magnitude

higher than the lowest concentration of total tetracyclines from JX3 soil (5.4 ± 1.7 $\mu g \cdot kg^{-1}$ dry soil).

Previous studies have shown that the highest average concentrations of TC and CTC in surface soil (0−10 cm) after fertilization with liquid manure were 86.2 and 6.6 $\mu g \cdot kg^{-1}$ dry soil, respectively (*41*), which is comparable to TC (95.3 $\mu g \cdot kg^{-1}$ dry soil) observed in the present study but lower than CTC (33.2 $\mu g \cdot kg^{-1}$ dry soil). We hypothesized the difference in the highest average concentrations of CTC in surface soil between this and previous studies might be associated with the initial concentrations of parent CTC in the manure waste and different ways of applying manure waste into soil as well as environmental factors. It is known that environmental factors such as pH, redox, and light conditions could influence the abiotic degradation of TCs in environments (*14*). It has been reported that the temperature could influence the persistence of CTC in soil (*41*). In the swine wastewater from concentrated swine feeding plants in Beijing, the tetracyclines (TC, OTC, CTC) have been detected showing a concentration range of 1.1−32.7 $\mu g \cdot L^{-1}$ (*9*), generally lower than those detected in Beijing samples in the present study. This is possibly due to the limited stability of the parent TCs in aqueous solution but high sorption to solid matrices (*42*). In domestic wastewater and surface waters in Beijing, the tetracyclines and their degradation products (ranging from 1.9 to 72.5 $ng \cdot L^{-1}$) (*14*) were much lower than those detected in the present study. These results are also consistent with the fact that tetracyclines are mainly applied in animal industry in China (*14*).

Responses of tet Genes to Environmental Factors. A bivariate correlation analysis was performed between total *tet* gene copy numbers (sum of the four genes, *tet*M, *tet*O, *tet*Q, and *tet*W) and measured physicochemical and biological soil data (Table S5) to assess the influence of other environmental factors on absolute *tet* gene copy numbers (Figure S2, $P < 0.05$). The strongest positive correlation with *tet* gene copies were 16S rRNA gene copies ($r^2 = 0.65$, $P < 0.01$) (Figure S2B). In addition, total *tet* gene copies were also significantly correlated with organic matter in soil ($r^2 = 0.46$, $P < 0.05$) (Figure S2C), which could be attributed to the significant relationship between 16S rRNA gene copies and organic matter (data not shown). Recent data have shown a negative relationship between *tet* genes and pH (*25*); however, there is no significant relationship between total *tet* gene copies and pH ($r = -0.14$, $P > 0.05$) in this study, and the reasons are not clear.

A bivariate correlation analysis further showed that the levels of total *tet* gene copies were significantly correlated to the total concentrations of tetracyclines with an r^2 value of 0.45 ($P < 0.05$) (Figure S2A). Although this relationship is not as strong as that in other studies (*21*), similar results were also found in Peak's study, and they hypothesized the weak correlation was possibly related to different environmental fate and transport mechanisms of resistance genes versus tetracycline after release (*24*). However, Pei and colleagues did not find a significant correlation between the concentrations of antibiotics and resistance gene numbers in environment (*22*). Anyway, the results in the present study are consistent with the hypothesis that resistance genes would be positively selected after greater exposure to TCs.

Considering the frequent usage of TCs in swine industry and its poor absorption by animals, the chances of bacteria in environment exposure to TCs highly increased. The resistance selection process can occur in gastrointestinal tracts of animals and in a manure waste holding system (*1*). So the presence of *tet* genes in soils may be the result of the migration of tetracycline-resistant bacteria or transmission of resistance genes by horizontal gene transfer from irrigation of waste lagoon water or from swine manure (*11*). It has been identified that piggery manure is a reservoir for transferable antibiotic resistance plasmids (*6*). Another resistance selection process is in soils after land application of waste lagoon water or manure containing TCs residues. It has been reported that TCs released to soils were strongly absorbed in various soil types and not to be readily degradable, combined with its constant input through manure waste or waste lagoon water, they can potentially provide a concentrated environment where alterations in local environmental microbiota can occur (*10*), including selection of resistant mutants in susceptible species, changes in the distribution of resistance genes present in gene-transfer elements, and selection of resistant species (*5*). Soil bacteria in close contact to manure or pigsty environment may thus have an important role in horizontal spread of resistance (*20*) as a huge environmental reservoir.

Cloning and Phylogenetic Analysis of tetM genes. A total of 191 clones for *tet*M from 9 libraries (BJ1, BJ2, BJ3, TJ1, TJ2, TJ3, JX1, JX2, JX3) were sequenced successfully. BLAST searches of the GenBank database confirmed that all of the sequenced clones matched known *tet*M genes, with sequence identities ranging from 98% to 100%. Phylogenetic trees of *tet*M gene sequences and related NCBI sequences are shown in Figure S3. Results showed at least five types of *tet*M existed in the soil samples. The most common type is the type 1 *tet*M present in a total of 117 clones (61%), which was identical to the sequences of *tet*M found in various plasmids, transposons, conjugative transposons, and chromosomes of both gram-negative and gram-positive bacteria. Compared to other genotyping, our type 1 *tet*M correspond to Mekong type 1 *tet*M by Kobayashi et al. (*28*). The type 5 *tet*M detected in 59 clones (31%) revealed high sequence identity (⩾99%) to the corresponding sequence in *Streptococcus agalactiae* (accession no. GU318219), *Streptococcal* conjugative shuttle transposon Tn1545 (accession no. X04388), and *Streptococcus pneumoniae* partial transposon Tn1545 (accession no. AM889142). The sequence of type 4 *tet*M in two clones (1%) was more closely related to the above three sequence types. Nine clones (5%) contained the type 2 *tet*M from *Staphylococcus aureus* transposon Tn916-like (accession no. EU918654) and *Streptococcus pneumoniae* (accession no. AY466395), and four clones (2%) contained the type 3 *tet*M from *Streptococcus pneumoniae* (accession no. EF472561).

It is known that alleles of *tet*M are widely distributed in a broad range of genera, and their transfer is presumably mediated by conjugative transposons Tn916-Tn1545 family and Tn5251 and large conjugative plasmids (*16*). However, further analysis is still needed to confirm the association of *tet*M gene with mobile genetic elements.

This study clearly demonstrates the presence of high levels of tetracycline residues and tetracycline resistance in soil environments adjacent to these swine feedlots, raising concerns that the overuse of antibiotics in the livestock system contributes to the resistance reservoir in the surrounding environment, through potential pathways such as waste lagoon seepage, irrigation of land with waste lagoon water, and land application of manures. Bivariate correlation analysis confirmed that the absolute *tet* gene copies in soil were not only strongly correlated with environmental factors such as ambient bacterial 16S-rRNA gene copies and organic matter but also significantly correlated with the concentrations of tetracycline residues in soil, which possibly indicated that resistance genes would be positively selected after greater exposure to TCs. The phylogenetic analysis on *tet*M revealed that most genotypes of *tet*M were associated with various conjugative transposons and plasmids, which may allow wider dissemination of *tet*M gene among numerous bacteria in environment, even in pathogens. However work is still needed to investigate the potential host range of *tet* genes

in soil environments, which may have significant implication for public health as pathogens carrying resistance genes are more dangerous. Although it is difficult to build a direct link between environmental resistance and public health concerns, the results in the present study highlight that soil plays an important role in the spread of antibiotic resistance and is a huge environmental reservoir.

Acknowledgments

This project was financially supported by the Ministry of Science and Technology, China (2007CB407304) and Chinese Academy of Sciences (KZCX1-YW-06-03). We also acknowledge the financial support of the International Scientific Cooperation Key Program of Zhejiang Province, China (2009C14026). We thank Mrs. Hongmei Zhang for providing soil samples from Jiaxing and Drs. Limei Zhang and Jupei Shen for many helpful instructions. We thank Professor Jianying Hu and Dr. Ai Jia for help in measuring tetracyclines. We thank Dr. Paul Williams for proofreading the manuscript.

Supporting Information Available

Three locations in China from which soil samples were originally collected (Figure S1); primers and PCR conditions used in this study (Table S1); detection of *tet* genes in soils around nine swine feedlots (Table S2); copies of *tet* genes in soil at different sites, normalized to sample volume (Table S3); copies of *tet* genes in soil at different sites, normalized to ambient 16S rRNA gene copies (Table S4); the mean chemical and biological conditions of each sample (Table S5); relationship between total *tet* gene copy numbers and measured physicochemical and biological soil data (Figure S2); neighbor-joining phylogenetic tree of *tet*M gene sequences detected in nine soil samples (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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ES1007802