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# Phenotypic and genotypic characterization of antibiotic-resistant soil and manure bacteria adjacent to swine production facilities

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**Abstract:** This study aimed to identify phenotypic and genotypic resistance profiles of bacteria isolated from soil and manure samples obtained from a farm where tetracycline and tylosin were used extensively. Samples were collected from the manure and soil, before and after manure application. All of the 151 bacteria were identified based on BLAST results. Minimum inhibitory concentrations were determined by agar dilution and E-test methods. Class-specific primers were used to amplify 6 erythromycin and 7 tetracycline resistance ($Tc^r$) genes for 46 tetracycline- and 18 erythromycin-resistant isolates. $Tc^r$ gene sequence identity between different species of isolates was identified to investigate the dissemination of resistance genes. All of the 151 bacteria belonged to 4 different phyla: *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. The majority of the tetracycline-resistant isolates were present in *Pseudomonadaceae*. Twenty of the isolates, representing 14 different genera, were positive for $Tc^r$ genes. Only 2 isolates possessed the *erm*(Q) gene. This study probably is the first to describe the presence of tetracycline genes in members of the genera *Simplicispira* and *Agrococcus*. Results of the study suggest that the diversity of $Tc^r$ gene-carrying bacteria are increasing. Furthermore, tetracycline-resistant bacteria could be detected 3 months after the manure application.

**Key words:** Antibiotic resistance, susceptibility, soil, swine manure, tetracycline, erythromycin

## 1. Introduction

Bacteria that are responsible for human disease have developed resistance to most of the common antibiotics within the last 10 years. Hospitals emerge as the most convenient environment for the development and transfer of this resistance. On the other hand, there is a growing concern about animal husbandry being another favorable source for the development of antibiotic resistance with excessive antibiotic use for prophylaxis, chemotherapy, and growth promotion. It is estimated that about 40% of all antimicrobials are used in livestock, including poultry, in North America and Europe (1).

There is no doubt that the widespread use of antibiotics in animal husbandry, especially where manure is used as fertilizer, can lead to the introduction of antibiotics, resistant bacteria, and resistance genes to environmental compartments such as soil, groundwater, and surface water, and to food (2–7). Large amounts of antibiotics, almost as much as 75%, are being excreted from animals unaltered and end up in manure, and then they pass to soil when applied to fields (8). The antimicrobial activity of tetracycline and tylosin stayed the same when they were adsorbed completely into soils, suggesting that they still have the potential to cause the development of antibiotic-resistant bacteria in the environment (9). Thus, antibiotics may influence the selection of resistant bacteria in the environment (10,11). However, selection pressure of antibiotic persistence is not the only mechanism increasing the antibiotic resistance rate in environmental bacteria (5,12). Studies show that subinhibitory concentrations of antibiotics would likely enhance the frequency of resistance gene transfer (13,14). Horizontal gene transfer is recognized as playing an important role in spreading antibiotic resistance genes in soil microbial communities (14,15). Studies also suggest that manure-treated environments exhibit a wide range of bacteria hosting diverse genetic elements (14,16).

The aim of the present study was to investigate phenotypic resistance patterns of tetracycline- and erythromycin-resistant bacteria from manure and

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manure-applied soil and the genetic material encoding the resistance. Several tetracycline resistance ($Tc^r$) gene sequence similarities between different species of bacteria were also identified to investigate further dissemination of resistance genes.

## 2. Materials and methods

### 2.1. Sampling

The soil samples were collected from a harvested soybean field located on a commercial swine confinement facility (Site C). The facility operations and geology of Site C were described previously (2–4). For the purpose of therapy, preventing diseases, or growth promotion, chlortetracycline, penicillin, and tylosin are used at Site C. Sampling was performed 4 times: 1 day before manure application and the first day, 3 weeks, and 3 months after manure application. All 4 samples were placed in a sterilized bottle and refrigerated at 4 °C until analysis.

Manure samples consisted of approximately 2-L subsamples that were collected from 8 different locations on each side of the lagoon and pooled into 1 sample (17) in sterilized bottles and kept on ice in the field. They were refrigerated at 4 °C in the laboratory until analysis. Samples were collected just before spreading to the field from the pit and lagoon manure.

### 2.2. Isolation of bacterial species

Soil samples (1 g) were taken from each core subsample, homogenized with stirring in 100 mL of cold Winogradsky salt solution (WSS) at 200 rpm for 30 min, and serially diluted in WSS. From each of the $10^{-5}$–$10^{-8}$ dilutions, aliquots (100 µL) were spread on R2A agar (Becton Dickinson, NJ, USA) or diluted in nutrient broth solidified with agar (DNBA) plates. Bacteria were cultivated for 12 days at 24 °C in the dark. After that, bacteria were subcultured onto the medium and were supplemented with tetracycline and erythromycin (Sigma Chemical Co., St. Louis, MO, USA) at 20 µg/mL final concentration for selection of antibiotic-resistant bacteria. Although higher than the corresponding Clinical and Laboratory Standards Institute (CLSI) breakpoints, the 20 µg/mL concentration of tetracycline and erythromycin certainly gives a relatively more reliable estimate of resistance. All media were amended with 25 µg/mL of pimaricin (Calbiochem, CA, USA) to inhibit fungal growth. In order to isolate bacteria from manure samples tryptic soy agar medium (Merck, Darmstadt, Germany) was used as well.

### 2.3. Susceptibility testing

The minimum inhibitory concentrations (MICs) of tetracycline and erythromycin were determined by use of either agar dilution or E-test methods in accordance with CLSI guidelines (18) and E-test manufacturer (AB Biodisk, Solna, Sweden) specifications, respectively. As the breakpoint for resistance, the following were used: tetracycline at ≥16 µg/mL and erythromycin at ≥ 8µg/mL. *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 were used as quality control strains. Since gram-negative bacteria are naturally resistant to erythromycin, only gram-positives susceptibilities were determined against that antibiotic.

### 2.4. Isolation of total DNA

DNA was extracted from the manure and soil bacteria that were incubated in broth medium using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, CA, USA) according to the manufacturer's instructions.

### 2.5. Bacterial identification

Bacteria were identified by determining the nucleotide sequence of 16S rRNA gene fragments from each organism. We used polymerase chain reaction (PCR) to amplify the V3 region (*Escherichia coli* positions 341 to 534) of the rRNA gene using the forward primer 5′-CCTACGGGAGGCAGCAG-3′ and reverse primer 5′-ATTACCGCGGCTGCTGG-3′ (19). Amplicons were purified by using the QIAQuick PCR purification kit (QIAGEN, CA, USA) according to the manufacturer's instructions and were subsequently sent to the University of Illinois Keck Center for Functional and Comparative Genomics for sequencing. Sequences were analyzed online by using the BLAST family of programs from GenBank.

### 2.6. Genotyping of antibiotic resistance determinants

#### 2.6.1. PCR detection of *erm* genes

Eighteen bacteria representing all the gram-positive species from all sampling times were tested for the presence of *erm* genes by PCR. Class-specific primers were used to amplify each of the 6 erythromycin resistance genes [*erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(G), and *erm*(Q)] described previously (5). PCR was conducted using multiplex PCR master mix (QIAGEN). The reaction mixture contained 2.0 µM of each primer, 2X QIAGEN multiplex master mix, and 10 ng of template DNA in a total volume of 25 µL. Amplification of all genes began with denaturation at 95 °C for 15 min. Denaturation was followed by 27 cycles of 30 s at 94 °C, 90 s at the 60 °C annealing temperature, and 90 s of extension at 72 °C. A final extension of 72 °C for 10 min was used for all samples.

#### 2.6.2. PCR detection of $Tc^r$ genes

Forty-six bacteria representing all the gram-positive and gram-negative species from all sampling times were tested for the presence of *tet* genes by PCR. The class-specific primer sets used to amplify the $Tc^r$ genes in this study were developed in our laboratory and published previously (17,20). The target *tet* genes were 3 genes encoding tetracycline efflux pumps, *tet*(C), *tet*(H), and *tet*(Z), and 4 genes encoding ribosomal protection proteins (RPP): *tet*(M), *tet*(O), *tet*(Q), and *tet*(W). All PCR cycles for detecting the presence or absence of resistance genes

were based on the method described by Koike et al. (4). Furthermore, resistance genes were subsequently sent to the University of Illinois Keck Center for Functional and Comparative Genomics for sequencing.

**2. 7. Phylogenetic analysis**

The sequences for *tet*(C) and *tet*(Z) genes were aligned using ClustalX. Based on the maximum-likelihood method, sequences were compared, and then a phylogenetic tree was created using MEGA version 5.05. The *Aquifex aeolicus fusA* gene was used as the outgroup. Reference nucleotide sequences used in tree construction were obtained from the GenBank database.

## 3. Results

### 3.1. Cultivation of antibiotic-resistant organisms

A total of 151 bacterial isolates were obtained from soil, swine manure, and waste lagoons. BLAST results showed that the distribution of isolates was spread among several different biotypes exhibiting a rich diversity. The isolates representing 4 different phyla: *Proteobacteria* (n = 104), *Bacteroidetes* (n = 14), *Actinobacteria* (n = 20), and *Firmicutes* (n = 13). These isolates are affiliated with 23 families (15 families of gram-negative and 8 families of gram-positive bacteria) (Table 1).

The 125 soil-isolated organisms mainly belonged to gram-negative bacteria (86%). Eighty-two percent of those belonged to the phylum *Proteobacteria* and there were bacteria found in 3 classes of this phylum (alpha-, beta-, and gamma-*Proteobacteria*). Other isolated gram-negative bacteria are affiliates of *Bacteroidetes*. Gram-positive bacteria belonged to 2 different phyla, namely *Actinobacteria* and *Firmicutes*. The predominant bacterial isolates were identified as members of the family *Pseudomonadaceae*. This distribution is parallel with the normal distribution of soil bacteria (21). The 26 pit and lagoon manure-isolated organisms mainly belonged to gram-positive species (58%) and are affiliated with *Actinobacteria* and *Firmicutes*. The predominant bacterial isolates were identified as members of family *Microbacteriaceae*.

### 3.2. Determination of phenotypic antibiotic resistance

A high level of resistance (HLR) (>256 μg/mL MIC value) to the tetracycline and erythromycin among manure- and soil-isolated bacteria was detected. Manure-isolated bacteria have HLRs to tetracycline and erythromycin of 9% and 22%, respectively. Soil-isolated bacteria have HLRs to tetracycline and erythromycin of 24% and 32%, respectively. Among the species, the highest HLRs were

**Table 1.** Phylogenetic affiliation of isolates.

| Phylum | Class | Family | Isolates |
|---|---|---|---|
| *Proteobacteria* | alpha-*Proteobacteria* | *Bradyrhizobiaceae* | 7 |
| | | *Phyllobacteriaceae* | 4 |
| | | *Sphingomonadaceae* | 3 |
| | | *Rhodospirillaceae* | 3 |
| | | *Caulobacteraceae* | 2 |
| | | *Rhodobacteraceae* | 1 |
| | beta-*Proteobacteria* | *Beijerinckiaceae* | 1 |
| | | *Burkholderiaceae* | 31 |
| | | *Comamonadaceae* | 1 |
| | | *Pseudomonadaceae* | 32 |
| | gamma-*Proteobacteria* | *Xanthomonadaceae* | 13 |
| | | *Moraxellaceae* | 6 |
| *Bacteroidetes* | | *Flavobacteriaceae* | 10 |
| | | *Flexibacteraceae* | 2 |
| | | *Sphingobacteriaceae* | 2 |
| *Actinobacteria* | | *Microbacteriaceae* | 8 |
| | | *Mycobacteriaceae* | 4 |
| | | *Corynebacteriaceae* | 2 |
| | | *Nocardioidaceae* | 2 |
| | | *Streptomycetaceae* | 4 |
| *Firmicutes* | | *Enterococcaceae* | 6 |
| | | *Bacillaceae* | 3 |
| | | *Staphylococcaceae* | 4 |

found in soil-isolated *Burkholderia* sp. and manure-isolated phylum *Actinobacteria*.

### 3.3. PCR detection of erythromycin resistance genes from soil and manure

All 18 erythromycin-resistant soil and manure bacterial isolated harbored only 2 *erm*(Q) genes, which belonged to *Staphylococcus simulans* and *Enterococcus* sp., and they were isolated from manure. In addition, *S. simulans* with the *erm*(Q) gene also had *tet*(W), *tet*(O), and *tet*(M) genes; *Enterococcus* sp. also had the *tet*(W) gene (Table 2).

### 3.4. PCR detection of $Tc^r$ genes from soil and manure

The 46 $Tc^r$ bacterial isolates were examined for the presence of 7 types of $Tc^r$ determinants, since these genes were frequently determined in our previous studies (2,17). PCR analysis indicated that 43% of the isolates, representing 14 different genera and having MIC ranges between 16 and 256 µg/mL, contained at least 1 of 3 of those 7 $Tc^r$ genes. They represented manure and soil isolates collected from all of the sampling times (Table 2). The remaining resistant isolates did not contain any of the $Tc^r$ determinants studied.

**Table 2.** Phenotypic and genotypic characterization, antibiotic susceptibility, and the closest sequence match of known phylogenetic affiliations of the bacterial isolates from manure and soil.

| Isolates | GenBank acc. no. | Closest relative | % similarity | Antibiotic susceptibility (µg/mL) | | *Tet* gene |
|---|---|---|---|---|---|---|
| | | | | *Erm* | *Tet* | |
| N14G1-1 | DQ137854 | *Pseudomonas* sp. | 99 | ND | 160 | |
| N8G3-1 | AY512608 | *Pseudomonas* sp. | 99 | ND | 128 | C |
| S2G1-1 | AY263484 | *Pseudomonas* sp. | 98 | ND | 64 | C |
| N8G3-2 | AY512608 | *Pseudomonas* sp. | 97 | ND | 128 | |
| S8G3-1 | AM161157 | *Pseudomonas putida* | 97 | ND | >256 | |
| N8G3-3 | AY512608 | *Pseudomonas* sp. | 92 | ND | 128 | |
| S8G2-1 | AM263521 | *P. anguilliseptica* | 99 | ND | 256 | |
| N14G-1 | AY267503 | *Stenotrophomonas* sp. | 99 | ND | 160 | |
| N8G-1 | AY379973 | *Stenotrophomonas* sp. | 99 | ND | >256 | |
| S6G2-1 | AY677090 | *Burkholderia pyrrocinia* | 100 | ND | 96 | |
| S6G2-2 | AY677090 | *B. pyrrocinia* | 99 | ND | >256 | |
| N14G1-2 | AY677090 | *B. pyrrocinia* | 99 | ND | >256 | |
| N14G-2 | AY677090 | *B. pyrrocinia* | 99 | ND | 160 | Q |
| N3G1-1 | AY677090 | *B. pyrrocinia* | 99 | ND | >256 | C, Z |
| N3G1-2 | HM587717 | *Burkholderia* sp. | 98 | ND | >256 | |
| N8G3-4 | AY741361 | *B. cepacia* | 99 | ND | 128 | |
| N8G3-5 | AY616143 | *B. stabilis* | 98 | ND | 128 | O |
| N8G1-1 | AY677090 | *B. pyrrocinia* | 98 | ND | >256 | H |
| N14G-3 | AY673130 | *Burkholderiaceae bacterium* | 97 | ND | >256 | C |
| N3G-1 | AB247235 | *B. cepacia* | 93 | ND | 16 | |
| PM-1* | EU434565 | *Simplicispira* sp. | 100 | ND | 32 | C, H, O |
| N14G3-1 | AY803987 | Beta-*proteobacterium* | 97 | ND | 128 | |
| S6G3-1 | AY803987 | Beta-*proteobacterium* | 97 | ND | >256 | |
| N14G1-3 | AY847284 | *Acinetobacter baumannii* | 100 | ND | 160 | C |

**Table 2.** (Continued).

| | | | | | | |
|---|---|---|---|---|---|---|
| PM-2 | DQ083490 | *Acinetobacter* sp. | 98 | ND | 16 | |
| N3G-2 | AY074793 | *Lysobacter* | 94 | ND | >256 | C |
| N8G-2 | AB245367 | *Dyella ginsengisoli* | 96 | ND | >256 | O |
| S8G2-2 | AY580798 | *Rhodocista* | 96 | ND | >256 | |
| S8G3-2 | AF208516 | *Bradyrhizobium elkanii* | 98 | ND | 64 | O |
| N8G1-2 | AY466722 | *Chryseobacterium joostei* | 100 | ND | 160 | |
| N3G-2 | AY466722 | *C. joostei* | 99 | ND | >256 | Q |
| N14G1-4 | AY167564 | *Flavobacterium* sp. | 96 | ND | >256 | |
| S6G3-2 | DQ339596 | *Flavobacterium* | 96 | ND | 64 | O |
| N3G1-3 | DQ448781 | *Mycobacterium* sp. | 99 | 160 | 160 | |
| N14G1-5 | DQ448781 | *Mycobacterium* sp. | 100 | >256 | 160 | |
| N8G3-6 | AY673418 | *Flexibacteraceae bacterium* | 98 | >256 | >256 | |
| N8G-3 | AY822606 | *Streptomyces cheonanensis* | 98 | ≤8 | 16 | |
| PM-3 | AJ971910 | *Streptomyces* sp. | 97 | 160 | 160 | Z |
| N14G2-1 | DQ026672 | *Streptomyces atrovirens* | 100 | 64 | 32 | Z |
| N8G1-3* | DQ232611 | *Agrococcus* sp. | 100 | >256 | >256 | C |
| PM-4 | AY126228 | *Staphylococcus saprophyticus* | 98 | >256 | 160 | |
| PM-5** | AY026056 | *S. simulans* | 100 | >256 | 128 | M, O, W |
| N8G1-4 | AF169529 | *Bacillus sp.* | 100 | 160 | 160 | |
| PM-6** | DQ305313 | *Enterococcus sp* | 99 | 256 | >256 | W |
| PM-7 | AY371450 | *Microbacterium* sp. | 100 | 160 | 160 | Z |
| N8G1-5 | DQ448707 | *Microbacterium* sp. | 100 | 256 | 160 | |

*: Genus in which *tet* genes were found for the first time.**: These strains also have the *erm*(Q) gene.
ND: Not determined (only gram-positives MIC values determined.), *erm*: erythromycin, *tet*: tetracycline.
Isolates are identified by a sampling site and time abbreviation (N: north; S: south; G: before manure application, G1: 1 day after manure application, G2: 3 weeks after manure application, G3: 3 months after manure application; PM: pit manure).

Among all tetracycline-resistant bacterial isolates, $Tc^r$ genes encoding efflux pumps were detected in 12 isolates and RPP encoding genes were detected in 9 isolates. The most prevalent tetracycline gene identified was *tet*(C), contributing to 8 (40%) of all the $Tc^r$ isolates positive for tetracycline genes, and 7 were identified as members of the phylum *Proteobacteria*.

In this study, to the best of our knowledge, we found that 2 new genera contained at least 1 $Tc^r$ gene. For example, we found an *Agrococcus* sp. that possessed *tet*(C), and in the same strain of *Simplicispira* sp. *tet*(C), *tet*(H), and *tet*(O) were identified. In addition to finding new genera that carry $Tc^r$ genes, another discovery was *Enterococcus* sp. possessing the *erm*(Q) gene as a new genus that carries this gene (Table 2). Furthermore, 10 of them were previously documented as being $Tc^r$ bacterial genera but we found new $Tc^r$ genes in them. Among those in gram-negative bacteria, we identified the *tet*(Q) gene in *Chryseobacterium joostei*; *tet*(C) gene in *Acinetobacter and Lysobacter*; *tet*(O) gene in *Dyella*, *Bradyrhizobium*, and *Flavobacterium*; and *tet*(O), *tet*(H), *tet*(C), and *tet*(Q) genes in *Burkholderia*. In gram-positive bacteria, the *tet*(Z) gene was found in *Streptomyces*, the *tet*(M) and the *tet*(O) genes in *Staphylococcus*, and the *tet*(W) gene in *Enterococcus* (Table 2). Multiple different tetracycline resistance genes were present in single bacterial isolates, which were

*Burkholderia pyrrocinia*, *S. simulans*, and *Simplicispira*. *B. pyrrocinia* has efflux genes *tet*(C) and *tet*(Z); *S. simulans* has RPPs *tet*(W), *tet*(O), and *tet*(M); *Simplicispira* sp. has *tet*(C), *tet*(H), and *tet*(O). While *Staphylococcus* sp. has been known to carry $Tc^r$ genes in these combinations, the combination of genera *Simplicispira* and *Burkholderia* is new.

**4. Discussion**

While the antibiotic resistance of clinical microorganisms has long been studied, the consequences of the existence of the antibiotics in nonclinical environments have only recently drawn attention. Because of the excessive use of antibiotics at these sites, one of the most convenient environments for studying the impact of the antibiotic existence in natural settings on environmental isolates is surely the area surrounding swine farms.

Tetracycline and tylosin were the first 2 antibiotics in use on swine farms for therapy, prophylaxis, and growth promotion. Tetracycline is a broad spectrum antibiotic and bacterial resistance to tetracycline is the most common form of antibiotic resistance. There are 43 $Tc^r$ genes currently described mediating resistance by different mechanisms such as efflux proteins, RPPs, and enzymatic inactivation (22). Erythromycin is a macrolide antibiotic reserved only for human use. It acts by binding to the 50S ribosomal subunit like tylosin does. Erythromycin resistance genes have been shown to confer resistance to tylosin.

In this study, resistance to tetracycline and erythromycin (only gram-positive bacteria were tested) have been detected in each species/genus, which are affiliated with 23 families, isolated from both soil and manure based on the sequencing and BLAST results. Furthermore, the erythromycin HLR rate was extremely high in soil- and manure-isolated bacteria, detected as 22% and 32%, respectively. These results could suggest that this broad diversity is perhaps the consequence of overuse of tetracycline and tylosin antibiotics. Erythromycin genes might be selected when exposed to any of the $MLS_B$ drugs, in this case to tylosin. For this reason, while the use of erythromycin has been limited to human consumption, the cause of high erythromycin resistance levels is potentially the tylosin administration in animal production (4,23). Furthermore, it is known that resistance genes for different antibiotics are carried in the same organism and in the same genetic elements. This could lead to the development of resistance due to the co-selection of one antibiotic for another. This phenomenon was specifically observed between macrolide and tetracycline antibiotic resistance (24–26). One study showed that tetracycline use in clinical *S. pyogenes* isolates is considered to be a factor contributing to the increase of macrolide resistance (27). In our study, the presence of the high-level frequency of $Tc^r$ observed with erythromycin resistance in bacteria isolated from the environmental compartments suggested that there might be a co-resistance. In this respect, the combined use of tetracycline and $MLS_B$ antibiotics in concentrated animal feeding operations should also be considered a driving factor for high frequency of erythromycin resistance in environmental isolates.

In this context, although high erythromycin resistance rate was detected, it is also interesting to note that we have determined only 2 *erm*(Q) genes in isolates that belonged to *Staphylococcus simulans* and *Enterococcus* sp. from the manure. It was noticeable that these 2 *erm*(Q) genes also exist in bacteria that carry $Tc^r$ genes. Tetracycline genes were detected in 20 out of 46 isolates. However, a reason for the lack of detection of these genes might be that we screened for only 6 of the 41 known rRNA methylase genes and 7 of the 43 known *tet* genes missing the other described erythromycin and tetracycline genes (22). Most probably they contained other resistance genes not examined in this study.

Diversity of $Tc^r$ genes carrying bacteria has been increasing rapidly. While only 39 genera carried these genes in 2001, the number rose to 115 in 2005 (28). In 2007, Kobashi et al. identified 13 other new genera that were determined to carry tetracycline genes, and Macauley et al. found 5 other new genera that carry tetracycline efflux genes (29,30). We found 2 new genera that were identified for the first time as carrying the *tet*(C) gene. Along with the 10 genera that were known to be $Tc^r$, new genes have been found in this study.

It was revealed in this study that both $Tc^r$ mechanisms existed in bacteria isolated from manure and soil, before and after the manure treatment. The results of the study also showed that dissemination of 7 $Tc^r$ genes was present adjacent to swine production facilities at least 3 months after manure application to soil.

The maximum-likelihood tree constructed by using $Tc^r$ genes encoding efflux proteins genes showed that all 6 of the *tet*(C) alleles and 4 of the *tet*(Z) alleles were grouped separately irrespective of where they were isolated (Figure). The detection of the closely related *tet*(Z) gene in bacteria of the gastrointestinal tract (*Streptomyces* sp.) and in bacteria typically connected with the soil habitat (*B. pyrrocinia* and *S. atrovirens*) at the same time indicates the possibility that they exist in a combined gene pool that produces gene transfer. Although it cannot be proven by this study alone, *Streptomyces* is considered the prominent strain carrying the tetracycline gene, as it was found that exactly the same *tet*(Z) genes were present in both soil and manure. Evidence for horizontal gene transfer might be the detection of the identical *tet*(C) allele in manure-isolated bacteria that were not shown to be carrying that

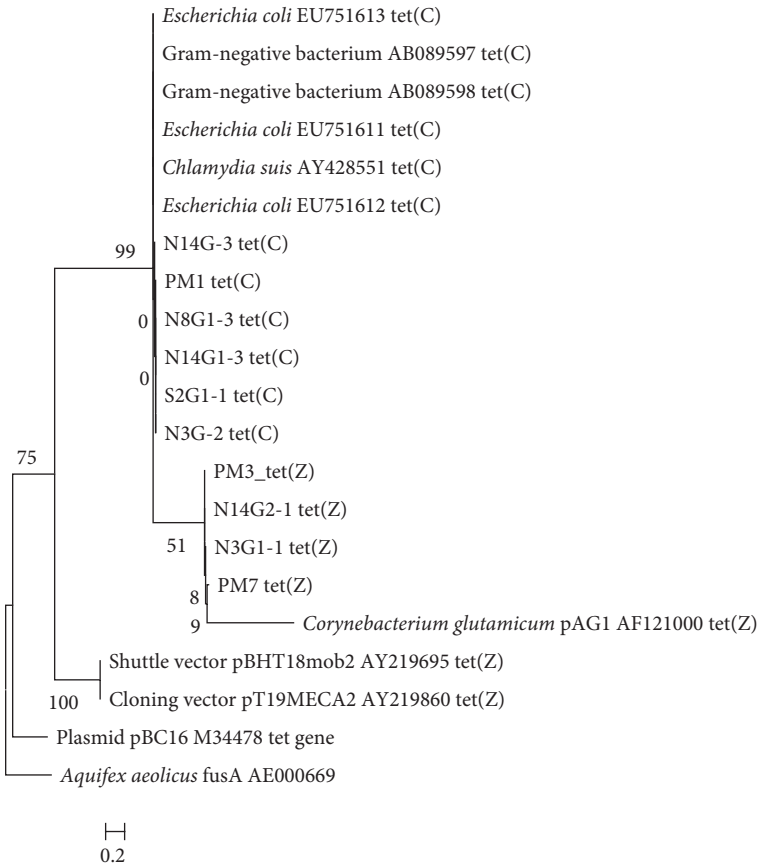


**Figure.** Phylogenetic analysis tetracycline resistance genes encoding efflux proteins performed with maximum-likelihood method. The scale bar represents nucleotide substitutions per sequence position. The *Aquifex aeolicus fusA* gene was used as the outgroup sequence.

gene before (*Simplicispira* sp.) and in soil-associated *Acinetobacter baumannii*, *Pseudomonas* sp., *Burkholderia pyrrocinia*, *Lysobacter* sp., and *Agrococcus* sp. It also indicates the high mobility of *tet*(C) and the increase of the diversity of tetracycline gene-carrying bacteria. It is also worth considering that bacteria causing infections in humans like *Pseudomonas* sp., *Acinetobacter* sp., and *B. cepacia* play a role in gene transfer. Although the detection of resistant bacteria in environmental samples relied heavily on cultivation techniques in this study, the detection of specific genes and their hosts is an important component in monitoring the migration of resistance gene carriers from swine farms to the environment.

It is known for sure the appearance and transfer of antibiotic resistance is a consequence of multiple interactions among antimicrobials, microorganisms, and the environment surrounding them. The increase in the number of antibiotic-resistant bacteria and genes is, on the other hand, an indication of our future failure in protection and treatment of infectious diseases in humans and animals. Certainly, there is need for more scientific data on the effect of use of antimicrobial substances in animal farming. In that respect, better understanding of the ecology of resistance should help us to design new treatment strategies.

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