Dissemination of veterinary antibiotics and corresponding resistance genes from a concentrated swine feedlot along the waste treatment paths

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ABSTRACT

Swine feedlots are an important pollution source of antibiotics and antibiotic resistance genes (ARGs) to the environment. This study investigated the dissemination of two classes of commonly-used veterinary antibiotics, namely, tetracyclines (TCs) and sulfonamides (SAs), and their corresponding ARGs along the waste treatment paths from a concentrated swine feedlot located in Beijing, China. The highest total TC and total SA concentrations detected were 166.7 mg kg^{-1} and $64.5 \text{ } \mu\text{g kg}^{-1}$ in swine manure as well as 388.7 and $7.56 \text{ } \mu\text{g L}^{-1}$ in swine wastewater, respectively. Fourteen tetracycline resistance genes (TRGs) encoding ribosomal protection proteins (RPP), efflux proteins (EFP) and enzymatic inactivation proteins, three sulfonamide resistance genes (SRGs), and two integrase genes were detected along the waste treatment paths with detection frequencies of 33.3–75.0%. The relative abundances of target ARGs ranged from 2.74×10^{-6} to 1.19. The antibiotics and ARGs generally declined along both waste treatment paths, but their degree of reduction was more significant along the manure treatment path. The RPP TRGs dominated in the upstream samples and then decreased continuously along both waste treatment paths, whilst the EFP TRGs and SRGs maintained relatively stable. Strong correlations between antibiotic concentrations and ARGs were observed among both manure and wastewater samples. In addition, seasonal temperature, and integrase genes, moisture content and nutrient level of tested samples could all impact the relative abundances of ARGs along the swine waste treatment paths. This study helps understand the evolution and spread of ARGs from swine feedlots to the environment as well as assess the environmental risk arising from swine waste treatment.

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1. Introduction

Antibiotics are widely used in livestock and poultry industries for disease control and growth promotion. It is estimated that 30–90% of the applied antibiotics cannot be digested or metabolized by animals, but excreted to the environment in their original forms with animal manure and urine (Sarmah et al., 2006). Tetracyclines (TCs) and sulfonamides (SAs) are two classes of the most commonly administrated veterinary antibiotics in concentrated swine feeding operations of China. In our previous studies, TCs and SAs were detected with high frequencies and concentrations in swine waste samples collected from 21 concentrated swine feedlots in Shandong province, China, with the highest concentration of 2.02 mg L^{-1} for oxytetracycline and $717 \text{ } \mu\text{g L}^{-1}$ for sulfamethazine in swine wastewater (Ben et al., 2013), as well as 764.4 mg kg^{-1} for chlortetracycline and 28.7 mg kg^{-1} for sulfamethazine in swine manure (Pan et al., 2011a).

China has the largest pork production in the world, which produces large amounts of manure and wastewater containing eutrophication elements and residual antibiotics every year. The presence of residual antibiotics could enhance the resistance level of microbial communities and promote the proliferation of antibiotic resistance genes (ARGs) in swine manure (Looft et al., 2012), livestock and poultry wastewater (Mckinney et al., 2010; Peak et al., 2007), farmland soil (Huang et al., 2013; Wu et al., 2010), and surface water (Graham et al., 2010; Luo et al., 2010). The residual antibiotics could promote the accumulation of ARGs even at subtherapeutic levels (Ghosh and Lapara, 2007). Nevertheless, most concentrated swine feedlots in China are lack of sufficient waste treatment facilities (Tong et al., 2009), especially for wastewater treatment (Ben et al., 2013), which makes them important reservoirs of antibiotics and ARGs (Cheng et al., 2013; Zhao et al., 2010; Zhu et al., 2013). As a result, environmental contamination of antibiotics and ARGs through unregulated discharge or use of swine wastes has been frequently reported. For example, Hu et al. (2010) found that the antibiotic concentrations in soil were closely related to the manure fertilization events. Wei et al. (2011) revealed that livestock and poultry wastewater could contaminate surrounding water systems as a major pollution source of antibiotics. Likewise, ARGs could also be easily disseminated in the environment through manure application (Heuer

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and Smalla, 2007; Peng et al., 2015) and wastewater discharge (Koike et al., 2007). However, up to date, studies on the transport of ARGs along manure and wastewater treatment paths in swine feedlots are very limited, and the correlations between residual antibiotics and ARGs need to be clarified. Moreover, although many studies have reported that besides antibiotics, other influential factors such as temperature (Pei et al., 2007), and moisture content (Chee-Sanford et al., 2009) and nutrient level (Séveno et al., 2002) of samples could also affect the behavior of ARGs, the relationships between these factors and ARGs along the waste treatment paths were not examined.

Therefore, a field investigation was conducted on the dissemination of TCs, SAs and their corresponding ARGs along both wastewater and manure treatment paths from a typical concentrated swine feedlot located in Beijing, China. Polymerase chain reaction (PCR) was applied to detect the target ARGs in collected samples, including four mutant dihydropteroate synthase genes reflecting the primary mechanism of sulfonamide resistance (Sköld, 2000), and sixteen tetracycline resistance genes (TRGs) covering three main mechanisms of tetracycline resistance (ribosomal protection protein (RPP), efflux protein (EFP), and enzymatic inactivation) (Roberts, 2012; Thaker et al., 2010). Then, the frequently detected TRGs and sulfonamide resistance genes (SRGs) were further quantified by quantitative PCR (q-PCR). In addition, the effects of various influential factors (i.e., seasonal temperature, and integrase genes, moisture content and nutrient level of collected samples) on the dissemination of ARGs along the waste treatment paths were also evaluated.

2. Materials and methods

2.1. Swine feedlot and sample collection

A concentrated swine feedlot located in Tongzhou district of Beijing, representative of swine feedlots in Northern China, was selected as the studied site with its waste (manure and wastewater) treatment illustrated in Fig. 1. The feedlot covered an area of 66,000 m² and kept about 6,000 pigs inside. TCs and SAs were two major classes of

antibiotics used in this feedlot as feed additives and injectable drugs. Swine wastewater, mainly composed of swine urine, swine bathing water and piggery manure washing water, was successively discharged to a collection channel, a settling pond, and finally a storage lagoon. As the frequencies of swine bathing and piggery washing were reduced in winter, much less wastewater was discharged into the storage lagoon than in summer. Swine manure and the settled solids in the settling pond were periodically gathered and transported to an adjacent composting plant located across the road. A cropland located to the east of the composting plant was fertilized with the mature compost. In this study, the following samples were collected along the manure treatment path: piglet manure (M1), sow manure (M2) and fattening pig manure (M3) from the swine houses; settled solids (WS1) from the settling pond; mature compost from the composting plant (S1); fertilized soil from the cropland (S2); and unfertilized soil from the road-side adjacent to the cropland (S3). Samples collected along the wastewater treatment path included: wastewater from the collection channel (W1); wastewater from the settling pond (W2); and wastewater (W3) and sediment (WS2) from the storage lagoon. The samples were collected in winter (February) and summer (August) of 2013, and at each sampling site, four subsamples were collected and then mixed into one composite sample. All samples were stored in ice-packed coolers and transported immediately to laboratory for pretreatment and analysis.

2.2. Chemical analysis

For the analysis of target antibiotics, the wastewater samples (i.e., W1, W2 and W3) were adjusted to pH 2.5–3.0 and centrifuged at 5000 × g for 15 min to separate the supernatant and suspended solids. Afterward, the liquid phase was subjected to solid phase extraction (SPE) (Ben et al., 2008), and the solid phase at the bottom of centrifuge tubes was collected and subjected to ultrasonic solvent extraction (USE) and SPE (Pan et al., 2011b). The detected concentrations in both liquid and solid phases were summed up as the total antibiotic concentration in a wastewater sample. The solid samples (i.e., M1, M2, M3, S1, S2, S3,

Fig. 1. Schematic diagram of waste treatment in the studied swine feedlot: wastewater collection channel (1); settling pond (2); storage lagoon (3); composting plant (4); and compost fertilized cropland (5).

WS1 and WS2) were also subjected to USE and SPE (Pan et al., 2011a). The concentrations of six SAs (i.e., sulfadiazine, sulfathiazole, sulfamethoxazole, sulfamethazine and sulfadimethoxine) and three TCs (i.e., oxytetracycline, chlortetracycline and tetracycline) in all the extracted wastewater and solid samples were determined in triplicate by ultra-high performance liquid chromatography and tandem mass spectrometry (UPLC-MS/MS, ACQUITY UPLC/Quattro Premier XE, Waters, USA) coupled with an Acquity Symmetry C18 column (50 × 2.1 mm, 1.7 μm, Waters, USA). Milli-Q water containing 0.2% formic acid (v/v) (A) and acetonitrile (B) were used as mobile phases at a total flow rate of 0.2 mL min⁻¹. The gradient elution program (time in min, % mobile phase B) was set as follows: (0, 5), (6, 8), (11, 10), (14, 13), (17, 22), (20, 30), (23, 35), (28, 100), (30, 100), (35, 10), and (41, 10). The MS parameters were optimized according to our previous study (Yuan et al., 2014). The limits of quantitation (LOQs) of the selected nine antibiotics were in the range of 2.45–6.68 μg kg⁻¹ for solid phase and 24.46–66.83 ng L⁻¹ for liquid phase, respectively.

The concentrations of chemical oxygen demand (COD), ammonia (NH₃-N), total nitrogen (TN) and total phosphorus (TP) were determined with a Hach DR5000 spectrophotometer (Loveland, USA) by Hach Methods 8000, 10031, 10072 and 8048, respectively. All wastewater samples were centrifuged at 5000 × g for 15 min and then the supernatant was filtered through 0.45-μm glass fiber filters (Millipore, USA) before analysis. For all solid samples, the concentrations of COD and ammonia were determined following ISO 14235:1998 and ISO/TS 14256-1:2003, respectively; and the concentrations of TN and TP were measured by the aforementioned Hach Methods after acid digestion (Raveh and Avnimelech, 1979). The major physico-chemical properties of the solid and wastewater samples were presented in Supplementary Data (Tables S1 and S2).

2.3. Detection and quantification of ARGs

For solid samples, 0.2 g of each fresh sample was used for DNA extraction. For wastewater samples, W1 and W2 (10 mL each) and W3 (100 mL) were filtered through 0.22-μm polyvinylidene fluoride membranes (Millipore, USA), and then the membranes were cut into pieces with pre-sterilized scissors and subjected to DNA extraction. All DNA extractions were performed with FastDNA SPIN kit for soil (MP-bio, USA). The concentration and quality of the extracted DNA were determined by spectrophotometry (Genequant 1300, GE healthcare, USA) and agarose gel electrophoresis, respectively. The extraction and subsequent analysis of DNA for each sample were performed in triplicate.

Sixteen TRGs, including eight EFP TRGs (*tetA*, *tetB*, *tetC*, *tetE*, *tetG*, *tetK*, *tetL* and *tetA/P*), seven RPP TRGs (*tetM*, *tetO*, *tetQ*, *tetW*, *tetS*, *tetT* and *tetB/P*), one enzymatic inactivation protein TRG (*tetX*), four SRGs (*sul1*, *sul2*, *sul3* and *sulA*), and three integrase genes (*int11*, *int12* and *int13*), were detected using PCR. The PCR reaction mixture (20 μL) consisted of 2 μL of 10× PCR buffer (Mg²⁺ Plus), 0.16 mM dNTPs, 0.2 μM each primer, 0.1 mg mL⁻¹ BSA, 1 U of *Taq* DNA polymerase (Takara, Japan), and 1 μL of DNA template. PCR reactions were run on a Life-pro TC-96 Thermal Cycler (Bioer, China) with the following program: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation for 45 s at 95 °C, annealing for 60 s at different temperatures and extension for 60 s at 72 °C, and finished with a final extension at 72 °C for 6 min. The primers and PCR conditions applied are listed in Table S3. Milli-Q water and pMD18-T vectors (Takara, Japan) carrying the corresponding genes were used in each run as the negative and positive controls, respectively. The PCR products were checked by electrophoresis in 1% agarose gel in 1× TAE buffer. The positive amplicon of each gene was ligated into pMD18-T vector and transformed into *Escherichia coli* DH5α (Takara, Japan). Positive clones cultured on the LB-agar were picked up and sequenced on a sequencing machine (ABI3300, Applied Biosystems, USA) to ensure their specificities.

Thereafter, ten TRGs (*tetA*, *tetC*, *tetG*, *tetL*, *tetA/P*, *tetM*, *tetO*, *tetQ*, *tetW* and *tetX*), two SRGs (*sul1* and *sul2*), and two integrase genes

(*int11* and *int12*), which were frequently detected in the tested samples, were quantified using the q-PCR method according to our former study (Wang et al., 2015). The detailed reaction programs, amplification efficiencies, and limits of detection for gene copy number are listed in Table S4.

2.4. Statistical analysis

All experimental data was statistically analyzed by SPSS 19.0 (IBM, USA). Paired samples *t*-test based on *p*-values (significant threshold = 0.05) was used to evaluate the significance of the differences among all samples. The correlations between ARGs and residual antibiotics, integrase genes, moisture content, and nutrient level of tested samples were evaluated by Pearson's bivariate correlation analysis.

3. Results and discussion

3.1. Antibiotic concentrations along manure and wastewater treatment paths

The concentrations of SAs and TCs in each sample were obtained by summing the detected concentrations of six SAs and three TCs, respectively. The variations of studied antibiotics along the manure treatment path are shown in Fig. 2a, with the individual antibiotic levels detailed in Table S5. In the manure samples (i.e., M1, M2 and M3), considerably higher TC concentrations were detected in a range of 0.58–166.7 mg kg⁻¹, indicating that TCs were commonly used in this

Fig. 2. Concentrations of tetracyclines (TCs) and sulfonamides (SAs) along manure treatment path (a) and wastewater treatment path (b). Sample symbols: piglet manure (M1), sow manure (M2), fattening pig manure (M3), mature compost (S1), fertilized soil (S2), unfertilized soil (S3), settling pond sediment (WS1), collection channel wastewater (W1), settling pond wastewater (W2), lagoon wastewater (W3), lagoon sediment (WS2); win: winter; sum: summer. Error bars represent the standard deviations (*n* = 3).

feedlot for disease prevention and growth promotion. The highest TC concentrations occurred in the sow manure (M2), which could be attributed to different dose levels and digestive abilities of various pig types (Haller et al., 2002; Pan et al., 2011a). The detected SA concentrations were much lower (from <LOQ to $64.5 \mu\text{g kg}^{-1}$) than those of TCs, with insignificant varying patterns among different manure samples, indicating that SAs were less used in this feedlot. Similarly, in the sediment of the settling pond (WS1), which mainly consisted of manure washed from swine houses, the TC concentrations were detected in a range of $30.3\text{--}35.8 \text{ mg kg}^{-1}$ while SAs were detected with much lower concentrations ($26.0\text{--}98.4 \mu\text{g kg}^{-1}$). Neither SAs nor TCs were detected in the mature compost (S1) and the subsequent soil samples (i.e., S2 and S3), indicating that these antibiotics could be effectively removed by the manure composting process.

As shown in Fig. 2b, both TC and SA concentrations in wastewater samples decreased along the wastewater treatment path from W1, W2 to W3. In the upstream wastewater samples (W1 and W2), their concentrations were higher in winter than in summer ($p < 0.05$), because lower temperatures tended to retard the degradation of antibiotics as well as less dilution by bathing and washing waters occurred in winter. In W1, the TC and SA concentrations were detected at $126.0\text{--}388.7$ and $0.44\text{--}7.56 \mu\text{g L}^{-1}$, respectively; and then decreased to $45.6\text{--}139.3$ and $0.1\text{--}1.65 \mu\text{g L}^{-1}$ in W2, respectively. In the storage lagoon, TCs were detected in summer ($0.18 \mu\text{g L}^{-1}$ in W3 and 1.01 mg kg^{-1} in WS2) but not detected in winter when less wastewater was discharged; by contrast, SAs showed less significant seasonal variations ($0.08\text{--}0.4 \mu\text{g L}^{-1}$ in W3 and $5.41\text{--}6.14 \mu\text{g kg}^{-1}$ in WS2). It could be attributed to the higher water solubility of SAs than that of TCs, which makes SAs more easily spread through wastewater (Chee-Sanford et al., 2009).

3.2. Detection frequencies of ARGs

Fourteen out of sixteen target TRGs (except *tetE* and *tetK*), three out of four target SRGs (except *sulA*), and two integrase genes (*int1* and *int2*) were detected by PCR in most of the collected samples (Fig. 3). *tetE* is often associated with non-conjugative plasmids, which may restrict its dissemination (Ng et al., 2001); *tetK* is usually related to methicillin-resistant *Staphylococcus aureus* (MRSA), a multidrug resistant pathogen (Roberts, 2012). Thus, it is not surprising for their absence. The detection frequencies of target ARGs ranged from 33.3% (integrase genes in summer) to 74.1% (TRGs in winter) along the manure treatment path and from 41.7% (integrase genes in summer) to 75.0% (TRGs in winter) along the wastewater treatment path. Overall, ARGs were more frequently detected in winter (50.0–75.0%) than in summer (33.3–67.2%), and this

Fig. 3. Detection frequencies of tetracycline resistance genes (TRGs), sulfonamide resistance genes (SRGs) and integrase genes (*intIs*) along manure treatment path (a) and wastewater treatment path (b).

difference was more obvious along the manure treatment path. Therefore, it seems that a higher moisture content and a lower temperature were favorable for the maintenance of ARGs, which agrees with what reported by another study (Chee-Sanford et al., 2009).

3.3. Abundances of ARGs along manure and wastewater treatment paths

Among all the detected ARGs, the most prevalent TRGs (i.e., four RPP TRGs, five EFP TRGs and *tetX*) as well as two SRGs (i.e., *sul1* and *sul2*) were selected for further quantitative analysis. Because the absolute abundance of ARGs was severely impacted by the total biomass extracted from each sample, the relative abundance of ARGs was used (i.e., normalized by the 16S rRNA gene abundance) in this study. The relative abundances of target ARGs (ARGs per 16S rRNA gene) in all tested samples ranged from 2.74×10^{-6} to 1.19, as detailed in Table S6.

The samples along the manure treatment path could be divided into the upstream and downstream groups. The upstream group (i.e., M1, M2, M3 and WS1) reflected the initial source of ARGs from pigs, and the downstream group (i.e., S1, S2 and S3) revealed the behavior of ARGs in the manure composting process and their subsequent transport to the environment. As shown in Fig. 4, in the upstream group, RPP TRGs were dominant with their relative abundances ranging from 1.47×10^{-3} to 1.19, probably because they are usually associated with some enteric bacteria (Aminov et al., 2001). The relative

Fig. 4. Relative abundances of ribosomal protection protein (RPP) TRGs and *tetX* (a), efflux protein (EFP) TRGs (b), and SRGs (c) along manure treatment path.

abundances of EFP TRGs, *tetX* and SRGs in the upstream group were in the ranges of 7.44×10^{-6} – 2.81×10^{-1} , 4.84×10^{-4} – 1.48×10^{-1} and 7.05×10^{-4} – 3.34×10^{-2} , respectively. Among the three types of manure (i.e., M1, M2 and M3), the maximal levels of TRGs were observed in the fattening pig manure (M3) rather than in the piglet manure (M1) that exhibited the highest TC concentrations, implying that long-term feeding with TCs could foster the proliferation of TRGs in the intestinal flora of pigs. In addition, the maximal levels of SRGs were found in M1 (2.11 – 3.34×10^{-2}), hinting that additional SAs were administered to the piglets for disease prevention as they are more susceptible to illness than sows and fattening pigs.

In the downstream group along the manure treatment path, most of the studied ARGs decreased, but different ARGs showed different variation patterns (Fig. 4). In the subsequent mature compost samples (S1), RPP and most EFP TRGs decreased dramatically, demonstrating a high efficiency of thermophilic composting in eliminating these ARGs. Conversely, *tetX* and SRGs were not eliminated significantly after manure composting and *tetX* even increased in winter. A former study found that biotic processes could contribute to the degradation of TCs during the manure composting process (Arikan et al., 2007). Hence, the increased *tetX* levels in S1 may suggest that *tetX* played a certain role in the biodegradation of TCs during manure composting. In the fertilized soil (S2) and unfertilized soil (S3), the relative abundances of target ARGs were in the ranges of 2.86×10^{-5} – 1.82×10^{-3} (RPP TRGs), 8.88×10^{-5} – 4.37×10^{-3} (EFP TRGs), 3.62×10^{-6} – 1.08×10^{-2} (*tetX*), and 4.44×10^{-4} – 1.82×10^{-2} (SRGs). RPP TRGs decreased more prominently than EFP TRGs along the manure treatment path, and EFP TRGs became more abundant in S1 and S2. *tetC* and *tetL* were the most prevalent TRGs in the downstream soil samples, with relative abundances of 4.38×10^{-4} – 4.37×10^{-3} .

Along the wastewater treatment path, the variations of target ARGs are shown in Fig. 5. The levels of target ARGs changed in different sampling seasons. For example, in the collection channel (W1) and the settling pond (W2), most ARGs were more abundant in winter (Table S6). This seasonal variation of ARGs was also reported by other studies (Mckinney et al., 2010; Yang et al., 2013), implying that low temperatures favored the maintenance of ARGs. Nevertheless, in the downstream lagoon wastewater (W3), all ARGs were less abundant in winter. This is probably due to two aspects: (1) less wastewater discharged in winter alleviated the contamination of the storage lagoon, which is consistent with the detected TC concentrations; and (2) the storage lagoon was stagnant, so the suspended solids in the lagoon wastewater (W3), including bacteria harboring ARGs, were likely to settle down to the sediment (WS2).

Similar to their behavior along the manure treatment path, most of the studied ARGs also decreased along the wastewater treatment path (Fig. 5). The RPP TRGs were more abundant in the upstream W1 and W2 samples (1.88×10^{-3} – 5.11×10^{-1}), but decreased obviously thereafter in the lagoon wastewater (W3) and sediment (WS2) (2.74×10^{-5} – 1.88×10^{-2}). Meanwhile, except *tetC*, other EFP TRGs and *tetX* also decreased from 1.42×10^{-5} – 2.61×10^{-1} to 1.09×10^{-5} – 7.57×10^{-3} , keeping approximately the same pace with the RPP TRGs. *tetC* was the only ARG that increased in the storage lagoon (2.86×10^{-3} – 2.04×10^{-1}) and became predominant there. The persistence of *tetC* during both manure and wastewater treatments was probably due to its frequent association with plasmids, which promoted its transfer between different bacteria (Roberts, 2012). The relative abundances of SRGs decreased from 1.36×10^{-2} – 1.86×10^{-1} to 1.76×10^{-4} – 5.51×10^{-2} along the wastewater treatment path, but were more abundant than most TRGs (Fig. 5c). As the SRGs also persisted in manure treatment (Fig. 4c), their high residues and stabilities are worthy of attention. *sul1* gene is often associated with *int11*, which could contribute to its dissemination (Mazel, 2006). *sul2* gene is frequently found in IncQ plasmids (a class of widely-distributed mobilizable plasmids with a small size and a broad host range), whose transfer could be easily mediated by other conjugative plasmids

Fig. 5. Relative abundances of RPP TRGs and *tetX* (a), EFP TRGs (b), and SRGs (c) along wastewater treatment path.

(Smalla et al., 2000). Moreover, bacteria carrying SRGs are unlikely to spend extra energy as dihydropteroate synthesis is necessary for their survival (Skögl, 2000). Therefore, it is not surprising to observe the high stabilities of SRGs during swine waste treatments.

Overall, although the studied ARGs generally decreased along both manure and wastewater treatment paths, their degree of reduction was more significant in the former case (Table S7). Wang et al. (2012) found that manure composting could better reduce the ARGs than lagoon treatment. Our results also show that the studied ARGs were more likely to disseminate through wastewater discharge than through manure composting and land application.

3.4. Correlation analysis

The presence of antibiotics exerts a direct selection pressure for the accumulation of ARGs. Along the manure treatment path, no significant correlation was observed between the total SA concentration and the total relative abundance of SRGs. However, with regard to TRGs, the total relative abundance of four RPP TRGs (i.e., *tetM*, *tetO*, *tetQ* and *tetW*) was strongly correlated with the total TC concentration in the upstream manure samples (i.e., M1, M2, M3 and WS1) (Pearson $r = 0.743$, $p < 0.05$) (Fig. 6a). Moreover, the dominance of the four RPP TRGs in the upstream samples indicated that ribosomal protection was the major

Fig. 6. Pearson's bivariate correlation between target ARGs and antibiotic concentrations in manure samples (M1, M2, M3, and WS1) (a) and wastewater samples (W1, W2, and W3) (b).

mechanism responsible for tetracycline resistance under the selection pressure of high TC concentrations.

Among the three main mechanisms responsible for tetracycline resistance, the EFP requires proton motive force to expel TCs out of bacterial membranes (Roberts, 2012); the enzymatic inactivation (*tetX*) needs oxygen and nicotinamide adenine dinucleotide phosphate to inactivate TCs (Yang et al., 2004); and the RPP needs guanosine triphosphate to trigger the release of TCs from their target sites (Li et al., 2013). All the three tetracycline resistance mechanisms need to consume energy. Regardless of other outside influences, bacteria with more energy-saving resistance patterns will be more competitive. Hence, ribosomal protection appears to be more efficient in protecting bacteria from the attack of TCs in the pig gut. In addition, some multi-drug efflux pumps, such as Mex and Acr in gram-negative bacteria, could be important for the tetracycline resistance (Piddock, 2006). They may assist EFP TRGs in the efflux of TCs, leading to poor correlations between TCs and EFP TRGs. Besides, the upstream manure samples held a similar environment to the pig gut (i.e., inadequate oxygen supply but sufficient nutrients), thus favoring the survival of anaerobic bacteria that often harbor RPP TRGs as well as contributing to the prevalence of RPP TRGs in these samples (Roberts, 2012).

Along the wastewater treatment path, a strong correlation between the total relative abundance of SRGs and the total SA concentration existed (Pearson $r = 0.946$, $p < 0.01$, Fig. 6b), while the correlation between the total TRGs and the total TCs was not significant. Similar results have been reported by previous studies (Gao et al., 2012; Luo et al., 2010; McKinney et al., 2010; Peak et al., 2007). It is seen that the relatively high water solubility and stability of SAs tend to promote the spread of antibiotic resistance and also contribute to the strong

correlation between SRGs and SAs in the aquatic environment (Chee-Sanford et al., 2009). By contrast, at low TC concentrations, many other factors (e.g., co-selection and cross-selection effects) could also affect the spread of TRGs in the aquatic environment, thus only a weak correlation between TRGs and TCs was observed along the wastewater treatment path (Gao et al., 2012).

The correlation analysis data of Log-transformed ARGs per 16S rRNA gene with other related influential factors are listed in Tables S8 and S9. Moisture is important for the survival of bacteria in the environment, and the superiority of some TRGs (*tetO* and *tetW*) in wastewater samples was reported by an earlier study (Storteboom et al., 2010). Our results show that *tetM*, *tetO*, *tetW* and *tetX* were significantly correlated with moisture content along the manure treatment path (Pearson $r > 0.65$, $p < 0.05$), indicating that moisture content was important for their maintenance. Nutrients can promote the horizontal transfer of ARGs in the environment (Séveno et al., 2002). In this study, the concentrations of COD, $\text{NH}_3\text{-N}$, TN and TP along the manure treatment path, and the concentrations of COD and $\text{NH}_3\text{-N}$ in the wastewater samples, were also correlated with some studied ARGs, especially RPP TRGs (Pearson $r > 0.53$, $p < 0.05$). These results suggest that nutrients could also favor the dissemination of some ARGs.

Integrases are greatly involved in the horizontal transfer of ARGs (Gillings et al., 2008; Mazel, 2006), and strong correlations between ARGs and integrase genes have been observed previously (Liu et al., 2012; Luo et al., 2010). Along the manure treatment path, *tetG*, *sul1* and *sul2* were correlated with *int11* (Pearson $r > 0.56$, $p < 0.05$), and *tetX*, *sul1* and *sul2* were correlated with *int12* (Pearson $r > 0.66$, $p < 0.01$); along the wastewater treatment path, *tetA*, *tetG*, *tetA/P*, *tetX* and *sul1* were correlated with *int11* (Pearson $r > 0.70$, $p < 0.05$). These results suggest that the integrase genes played an important role in the dissemination of target ARGs. *sul1* is typically located in the 3'-conserved area of class 1 integron and *tetG* gene cassette has also been detected in class 1 integron (Boyd et al., 2000; Mazel, 2006), which may explain their high correlations. The correlations between other studied ARGs and the integrase genes were probably because they were located in the same host group or gene elements such as conjugative plasmids and insertion sequence common region (Agersø and Sandvang, 2005; Toleman et al., 2006).

4. Conclusions

This study investigated the dissemination of two classes of commonly used veterinary antibiotics and their corresponding ARGs along swine waste treatment paths from a typical concentrated swine feedlot. Based on the experimental results, we can draw the following conclusions:

- The total TC concentrations reached up to 166.7 mg kg^{-1} in swine manure and $388.7 \text{ } \mu\text{g L}^{-1}$ in swine wastewater, which were about 10–1000 fold higher than the total SA concentrations (up to $64.5 \text{ } \mu\text{g kg}^{-1}$ in manure and $7.56 \text{ } \mu\text{g L}^{-1}$ in wastewater) in most collected samples. The antibiotic concentrations gradually decreased along both wastewater and manure treatment paths.
- The detection frequencies of target ARGs were higher in winter (50.0–75.0%) than in summer (33.3–67.2%). The relative abundances of target ARGs ranged from 2.74×10^{-6} to 1.19 and decreased along the waste treatment paths. Their degree of reduction was more significant along the manure treatment path.
- RPP TRGs were dominant in the upstream samples along the waste treatment paths, and decreased remarkably thereafter. EFP TRGs decreased relatively slower than RPP TRGs, and became dominant TRGs in the downstream samples. SRGs were generally more stable and abundant than most TRGs during swine waste treatments.
- In the upstream samples along the manure treatment path, RPP TRGs were significantly correlated with the total TC concentrations. Along the wastewater treatment path, a strong correlation between SRGs and SAs was observed. In addition, the moisture content and nutrient

level were significantly correlated with some ARGs, mostly RPP TRGs, indicating that besides antibiotics, other factors could also affect the dissemination of ARGs.

- Several ARGs (e.g., *tetG*, *sul1*, *sul2*) were significantly correlated with the integrase genes, indicating that integrons played an important role in the dissemination of ARGs along the swine waste treatment paths.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.envint.2016.04.020>.

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