Detection of sulfonamide resistant bacteria and resistance genes in soils

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Abstract. Manure application could accelerate the environmental dissemination of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in soils. In this study, the prevalence of sulfonamide resistant bacteria and resistance genes was investigated in agricultural soils to which organic manures had been applied in Tianjin, China. Anti-sulfonamide bacteria were found in the range of 3.29×10^4 to 1.70×10^5 CFU/g dry soil, occupying 1.5% to 2.2% of total viable counts. And *sul*I and *sul*II genes were detected in all sampling sites, with relative abundances of 5.69×10^{-5} to 6.95×10^{-4} and 4.28×10^{-4} to 1.25×10^{-3} respectively. No significant correlations between cultivable sulfonamide resistant bacteria and *sul* genes were found in this study. While *sul*I showed significant positive correlation with soil organic matter. Overall, the results highlight that soil plays an important role in resistance genes capture as the environmental reservoir.

1. Introduction

Manure is a major source of antibiotic pollution in the environment. Since many antibiotics are poorly absorbed by the animals and subsequently are excreted into the environment by urine or feces. The antibiotics tend to persist and accumulate in soils after repeated manure application. Residual antibiotics may exert selection pressure on environmental microorganisms, accelerating the environmental dissemination of antibiotic resistance. Bacteria have been shown to readily share genetic information by horizontal gene transfer (HGT) mechanism driven by mobile genetic elements, permitting the transfer of antibiotic resistance genes (ARGs) among environmental bacteria. Numerous antibiotic resistant bacteria (ARB) and ARGs have been widely detected in soil in the past few decades [1-4]. ARB and ARGs in soils have the potential to pose risks to human health, as susceptible pathogenic bacteria can become resistant by acquiring resistance genes in the environmental media [5]. Therefore, it is of great importance to investigate the behavior of ARB and ARGs in agricultural soils.

Sulfonamides are one of the most commonly used in livestock system. Sulfonamide-resistance genes (*sul* genes) were also observed with high levels of abundances in Tianjin area [6]. In this work, the prevalence of sulfonamide resistance was investigated in agricultural soils to which organic manures had been applied from four districts in Tianjin, China. Culture-dependent method was used to assess the resistance rate of bacteria in soils exposed to different sulfonamide concentrations. The

quantitative PCR (qPCR) was used to quantify *sul*I and *sul*II genes. Meanwhile, the relationship between ARGs, ARB and environmental factors was also investigated.

2. Materials and Methods

2.1. *Soil samples*

Soil samples were collected from agricultural fields to which organic manures had been applied. The samples were taken from Jixian, Xiqing, Wuqing and Dagang Districts (define as JX, XQ, WQ and DG respectively) in Tianjin, China, between March and May 2016. For each site, three subsamples were taken from 0 to 10 cm surface soil and were mixed to form one composite sample. Fresh samples were processed immediately for cultivation of ARB. The other samples were stored at -80°C before DNA extraction and chemical analysis. Weights of wet soils and oven-dry soils were measured to derive soil moistures. Soil pH was determined with a soil to water ratio of 1:2.5. Total carbon (TC) was measured by a by a TOC analyzer (TOC-VCPH, SHIMADZU). Organic matter (OM) was determined by the $K_2Cr_2O_7$ oxidation method. The physicochemical properties of soil samples are summarized in Table 1.

Table 1. Physicochemical properties of soil samples

Samples	Moisture (%)	pH	TC (mg/g)	OM (%)
JX	17.87±0.08	7.51±0.22	19.59±0.12	4.46±0.03
XQ	21.15±0.10	7.38±0.04	28.88±2.01	3.79±0.40
WQ	30.13±0.21	7.86±0.02	26.80±0.28	3.12±0.04
DG	7.67±0.01	8.14±0.06	25.48±1.26	3.75±0.24

2.2. *Detection of ARB*

Each soil sample was measured in duplicate to determine the numbers of ARB using colony forming units count method described by Chen et al. [5] with minor modifications. Briefly, 5.0 g of wet soil was mixed with 45 mL sterile saline solution (0.85% NaCl) and shaken vigorously on an oscillator at 200 rpm for 20 min. 100 μL of ten-fold serial dilutions for each suspension were spread onto a broth agar plates containing selected concentrations of antibiotics (20 and 50 μg/mL of sulfadiazine). Broth agar plates with no antibiotics was used to determine the total cultivable bacteria numbers.

2.3. *Detection of ARGs*

Total DNA was extracted from 0.5 g of soil (fresh weight) by using FastDNA SPIN kit for Soil (MP Blomedicals, LLC., France). The concentration and quality of the extracted DNA was determined by spectrophotometer analysis (NanoDrop ND-1000, Theromo Fisher Scientific). PCR assays were used for broad-scale screening of the presence/absence of *sul* genes, and to get the standards for subsequent qPCR analysis. All PCR assays were conducted in a Peltier Thermal Cycler (Bio-Rad). Primers and annealing temperatures are described in Table 2.

After PCR amplification, gel slices of an agarose gel containing the PCR products were excised, and purified using EasyPure Quick Gel Extraction Kit (TransGen). The purified PCR products were ligated into p-GEM T easy vector (Promega) and then cloned into *Escherichia coli* DH5α (Tiangen). Clones containing target gene inserts were picked and sequenced. If the gene inserts were verified as the object resistance genes using the BLAST alignment tool, clones that had right gene inserts were chosen as the standards for the subsequent qPCR. Plasmids carrying target genes were extracted with Plasmid Kit (TaKaRa). Two target genes (*sul*I and *sul*II) were quantified by qPCR using a SYBR-Green approach. 16S rRNA genes were quantified according to the TaqMan qPCR method [8]. 10-fold serial dilutions of a known copy number of plasmid carrying respective genes were generated to produce the standard curve. The qPCR mixtures (total volume, 20 μL) consisted of 10 μL of SYBR

Premix Ex Taq (TaKaRa), 0.3 μL of each primer (20 μM), 1.6 μL of template DNA and 7.8 μL of ddH_2O. The qPCR procedure was conducted using an iCycler IQ5 Thermocycler (Bio-Rad). The protocol was as the following program: 45 s at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at the annealing temperatures. Product specificity was confirmed by melting curve analysis (55–95°C, 0.5°C per read, 30 s hold). Each reaction ran in triplicate. The PCR efficiencies (96.7–105.1%) were examined to test for inhibition. R^2 values were more than 0.99 for all calibration curves.

Table 2. Primers and PCR conditions used in this study

Target genes	Primer	Sequences (5′—3′)	Amplicon size（bp）	Annealing temp (°C)	Reference
*sul*I	*sul*I-F	cgcaccggaaacatcgctgcac	163	56	[7]
	*sul*I-R	tgaagttccgccgcaaggctcg			
*sul*II	*sul*II-F	tccggtggaggccggtatctgg	191	61	[7]
	*sul*II-R	cgggaatgccatctgccttgag			
16S rRNA	1369F	cggtgaatacgttcycgg	123	56	[8]
	1492R	ggwtaccttgttacgactt			

2.4. *Data analysis*

Averages and standard deviations of all data were determined using Microsoft Excel, 2007. All statistical analyses were performed using SPSS version 19.0, and significant differences were determined by the *t* test. Bivariate correlation analyses were conducted to obtain Spearman's coefficients.

3. Results and Discussions

3.1. Occurrence of sulfonamide-resistant bacteria

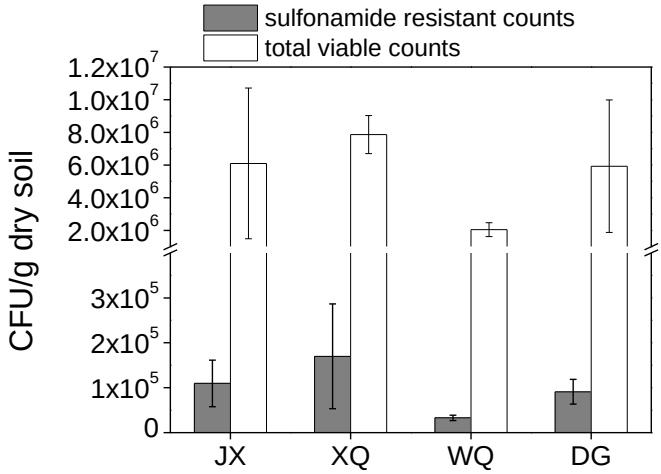

Figure 1. Total viable microbial counts and sulfonamide-resistant counts (resistant to 50 μg/mL of sulfadiazine) in soil samples.

As shown in figure 1, total concentrations of cultivable bacterial cells in soils ranged from 2.05×10^6 to 7.86×10^6 CFU/g dry soil. Cultivable live anti-sulfonamide bacteria were observed in all soil samples, ranging from 3.29×10^4 to 1.70×10^5 CFU/g dry soil. The relative abundances of sulfonamide-resistant strains (resistant to 50 μg/mL of sulfadiazine) observed in this study (1.5% to 2.2%) were much lower than those in wastewater-irrigated soils (approximately 10% to 20%) [5].

These resistant bacteria may come directly from the indigenous soil environment. They may also result from the stress of antibiotics in soil which needs to be further studied.

Meanwhile, the sulfonamide resistance rate of bacteria in soils exposed to different sulfonamide concentrations are investigated (figure 2). With the increase of sulfonamide concentration (from 20 to 50 μg/mL of sulfadiazine), the resistance rate of bacteria showed a decreasing trend (4.4%-9.4% at 20 μg/mL, 1.5%-2.2% at 50 μg/mL). Significant difference was observed in resistance rate under different sulfonamide concentrations ($P < 0.01$). But exposed to the same sulfonamide concentration, there is no significant difference in drug resistance of bacteria among different samples.

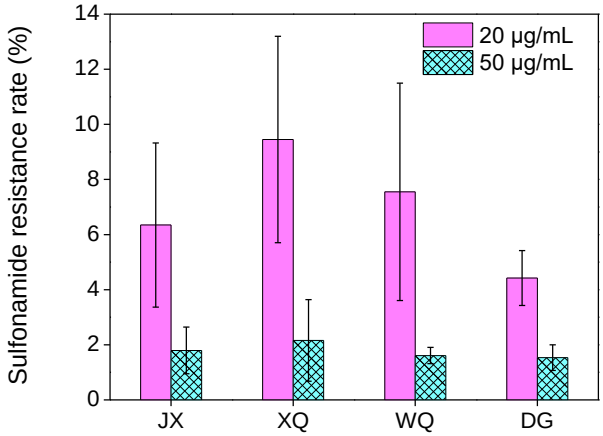

Figure 2. The sulfonamide resistance rate of bacteria in soil samples exposed to different sulfonamide concentrations (20 and 50 μg/mL of sulfadiazine).

3.2. *Determination of sulfonamide resistance genes*

Absolute gene copy numbers of two *sul* genes (*sul*I and *sul*II) in soil samples were presented in figure 3a. The absolute abundances of *sul*I genes in JX (1.58×10^7 copies/g dry soil) and XQ (1.82×10^7 copies/g dry soil) were comparable, but significantly higher than those in WQ and DG (10^6 copies/g dry soil). The highest abundance of *sul*II genes was occurred in WQ (1.32×10^8 copies/g dry soil), approximately 1 order of magnitude higher than those in other three samples. This could be related to the high 16S rRNA gene copies in WQ (1.58×10^{11} copies/g dry soil).

To minimize variance caused by different extraction and analytical efficiencies and differences in background bacterial abundances, the absolute number of all resistance genes were normalized to that of ambient 16S rRNA genes (figure 3b). The same general trends in gene abundance were seen in the normalized data relative to absolute data, since the total number of 16S rRNA gene copies was found to be relatively consistent among different sites at 10^{10} copies/g dry soil, except for WQ. The relative abundances (target gene/16S rRNA genes) of *sul* genes showed significant variation over sampling sites, ranging from 10^{-5} to 10^{-3}. In all sampling sites, the relative abundances of *sul*II (4.28×10^{-4} to 1.25×10^{-3}) were higher than those of *sul*I (5.69×10^{-5} to 6.95×10^{-4}). This observation is consistent with the results obtained in the aquaculture environment of Tianjin (relative abundances of 10^{-4} to 10^{-3} for *sul*II, and 10^{-5} to 10^{-4} for *sul*I) [6]. The total absolute and relative abundances of two *sul* genes (*sul*I and *sul*II) on average in different sites were in the range of 3.70×10^7 to 1.41×10^8 copies/g dry soil, and 8.91×10^{-4} to 1.95×10^{-3} respectively.

In this study, *sul*I and *sul*II genes were detected with high abundance, which were comparable to those in wastewater and reclaimed water irrigated soils in China [4, 5]. The high prevalence of *sul* genes could be related to residual antibiotics in soils introduced by manure application or wastewater irrigation. However, even in the absence of continuous selection of sulfonamides, *sul*I was found to be associated with persistent sulfonamide resistance in microorganisms [6]. Overall, the results highlight that soil plays an important role in resistance genes capture as the environmental reservoir.

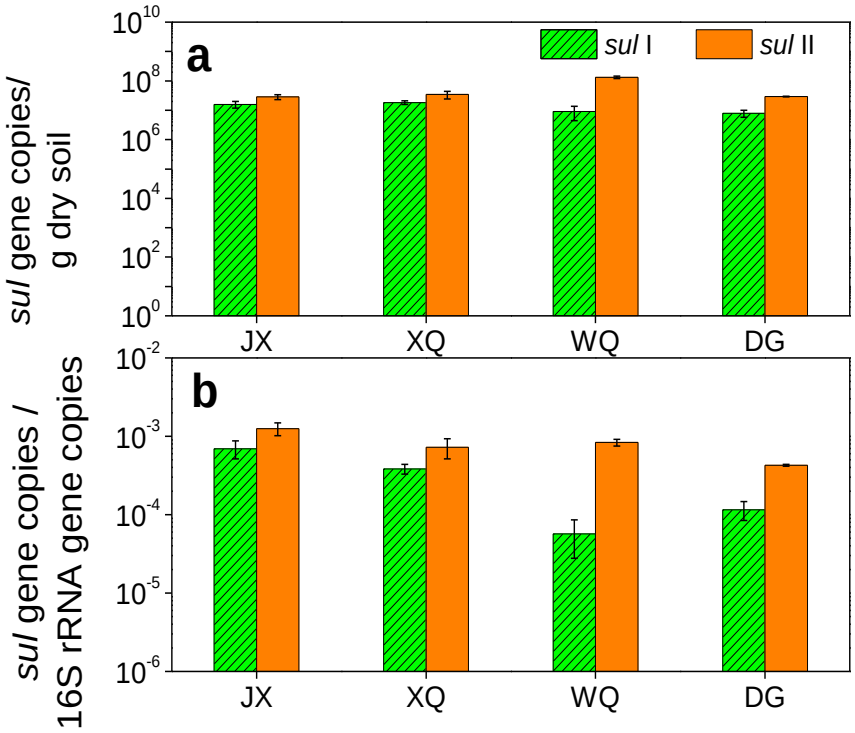

Figure 3. Detected levels of *sul* genes (*sul*I and *sul*II) normalized to (a) sample volume (copies per g dry soil) (b) copies of ambient 16S rRNA gene present in soil samples in different sites. Error bars indicate the standard deviation of three replicates.

Table 3. Correlation analysis of relative abundances of ARGs, ARB and environmental factors

	*sul*I	*sul*II	*sul*	pH	TC	OM	sulfonamide resistant bacteria
*sul*I	1.0	0.4	0.8	-0.6	-0.4	1.0**	0.8
*sul*II	0.4	1.0	0.8	-0.4	-0.4	0.4	0.0
sul	0.8	0.8	1.0	-0.8	-0.2	0.8	0.6
pH	-0.6	-0.4	-0.8	1.0	-0.4	-0.6	-0.8
TC	-0.4	-0.4	-0.2	-0.4	1.0	-0.4	0.2
OM	1.0**	0.4	0.8	-0.6	-0.4	1.0	0.8
sulfonamide resistant bacteria	0.8	0.0	0.6	-0.8	0.2	0.8	1.0

** represents statistical significance with $P < 0.01$; *sul* represents the sum of *sul*I and *sul*II genes.

3.3. *Relationship between ARGs, ARB and environmental factors*

According to the Spearman's correlation coefficients (Table 3), no significant correlations between cultivable sulfonamide resistant bacteria and *sul* genes were found in this study. This is not surprising given that only a very small fraction of soil microbes were targeted in cultivation. A considerable part of *sul* genes quantified could possibly exist in uncultivable bacteria. In addition, the relative abundance of *sul*I showed significant positive correlation with soil organic matter. This observation was in agreement with previous report [3]. But for *sul*II, no significant positive correlation was found with organic matter. Recent data have shown a significant negative relationship between ARGs and soil pH, since soil pH exerted a strong selection pressure on soil microbes and appeared to have a

pervasive effect on the abundance of bacteria [4]. However there is no significant relationship between *sul* genes and pH ($r = -0.8$, $P > 0.05$) in this study. This observation was in agreement with our previous report [3].

4. Conclusions

This study demonstrates the presence of high levels of sulfonamide resistance in agricultural soils in Tianjin area, raising concerns that the overuse of antibiotics in livestock system contributes to the environmental reservoir of resistance, through the potential pathways such as land application of manures. No significant correlations between cultivable sulfonamide resistant bacteria and *sul* genes were found in this study. But *sul*I showed significant positive correlation with soil organic matter. The relationship between ARGs and other environmental factors such as residual antibiotics, needs to be further studied. Overall, the results highlight that soil plays an important role in resistance genes capture as the environmental reservoir.

Acknowledgments

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References

[1] H. Lin, W.C. Sun, Z.L. Zhang, S.J. Chapman, T.E. Freitag, J.R. Fu, X. Zhang, J.W. Ma, Effects of manure and mineral fertilization strategies on soil antibiotic resistance gene levels and microbial community in a paddy-upland rotation system, Environ. Pollut. 211 (2016) 332-337.

[2] Y.G. Zhu, T.A. Johnson, J.Q. Su, M. Qiao, G.X. Guo, R.D. Stedtfeld, S.A. Hashsham, J.M. Tiedje, Diverse and abundant antibiotic resistance genes in Chinese swine farms, Proc. Natl Acad. Sci. USA. 110 (2013) 3435-3440.

[3] N. Wu, M. Qiao, B. Zhang, W.D. Cheng, Y.G. Zhu, Abundance and diversity of tetracycline resistance genes in soils adjacent to representative swine feedlots in China, Environ. Sci. Technol. 44 (2010) 6933-6939.

[4] F.H. Wang, M. Qiao, Z.E. Lv, G.X. Guo, Y. Jia, Y.H. Su, Y.G. Zhu, Impact of reclaimed water irrigation on antibiotic resistance in public parks, Beijing, China, Environ. Pollut. 184 (2014) 247-253.

[5] C. Chen, J. Li, P. Chen, R. Ding, P. Zhang, X. Li, Occurrence of antibiotics and antibiotic resistances in soils from wastewater irrigation areas in Beijing and Tianjin, China, Environ. Pollut. 193 (2014) 94-101.

[6] P. Gao, D. Mao, Y. Luo, L. Wang, B. Xu, L. Xu, Occurrence of sulfonamide and tetracycline-resistant bacteria and resistance genes in aquaculture environment, Water Res. 46 (2012) 2355-2364.

[7] R.T. Pei, S.C. Kim, K.H. Carlson, A. Pruden, Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG), Water Res. 40 (2006) 2427-2435.

[8] M.T. Suzuki, L.T. Taylor, E.F. DeLong, Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays, Appl. Environ. Microbiol. 66 (2000) 4605-4614.