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Quantification of residual antibiotics in cow manure being spread over agricultural land and assessment of their behavioral effects on antibiotic resistant bacteria

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HIGHLIGHTS

- TC (17.74 μg/kg), OTC (0.78 μg/kg), STZ (0.23 μg/kg) were detected in the topmost soil (collected at ground surface).
- *Pseudomonas* spp., *Arthrobacter* spp. and *Rhodococcus* spp. had persistent resistance to the 3 antibiotics tested by 16s rDNA.
- *Pseudomonas* spp. showed strong resistance to STZ, and *Arthrobacter* spp. and *Rhodococcus* spp. to TC and OTC by RT-qPCR.
- The concentrations of STZ, TC, and OTC degraded by ARB respectively dropped to 23.53%, 35.60% and 66.88%.

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ABSTRACT

Antibiotic resistant bacteria (ARB) in livestock manure used as fertilizer and spread over agriculture land, may pose a threat to the health of humans. Considering this, the concentrations of tetracycline (TC), oxytetracycline (OTC), and sulfathiazole (STZ) in the surface soil were quantified using LC-MS. These antibiotics have been used in livestock and are found in fertilizer produced from livestock excretions. Species of ABR were identified using 16S rDNA. Soil samples were collected at depths of 0, 7, and 15 cm from farmland in Incheon (South Korea). In the surface soil, three compounds were detected: TC (17.74 μg/kg), OTC (0.78 μg/kg), and STZ (0.23 μg/kg). However, except for STZ, antibiotics were not detected in the deeper samples. Overall, TC can form a chelated complex with cations, which consequently enhances its adsorption to the organic matter and metals in soil. This property can significantly reduce the mobility of TC (to lower than that of STZ). The result of 16S rDNA gene analysis indicated that *Pseudomonas* spp., *Arthrobacter* spp., and *Rhodococcus* spp. showed persistent resistance to the three antibiotics tested. DNA quantification results revealed strong resistance of *Pseudomonas* spp. to STZ, whereas *Arthrobacter* spp. and *Rhodococcus* spp. had resistance to TC and OTC. Antibiotics biodegradation suggested ability of ARB to grow in soil samples in presence of residual antibiotics during 13 days incubation. The concentrations of STZ, TC, and OTC reduced as much as 23.53, 35.60 and 66.88%, respectively.

1. Introduction

1.1. Popular antibiotics actively used in recent years

Pharmaceutical compounds such as human and veterinary medicines and cosmetics are new “pollutants” not encompassed within existing list of priority environmental pollutants. Pharmaceutical compounds are essential to have improved standard of living (Liu and Wong, 2013). The production of medicines in South Korea has annually increased by more than 5%, since 2006. For treatment and effective prevention of bacterial or virus infections, different classes of antibiotics such as tetracyclines (TCs), aminoglycosides, and sulfonamides (SAs) are frequently used in veterinary, livestock, and human medicine (Munir and Xagoraraki, 2007; Thiele-Bruhn, 2003). TCs and OTC are used to treat respiratory and sexually transmitted diseases inflammations in human. Oxytetracycline (OTC) is also indicated for the treatment of infectious diseases in fodder animals. TCs account for 50% of the total amount of antibiotics used in South Korea. STZ is another widely used antibiotics effective against bovine respiratory disease complex and a

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range of gram positive and gram negative pathogens (Chung et al., 2016). It is also actively used in fish farm and aquarium (Kim et al., 2008, 2010). TC, OTC and STC are detected by several researches and remained at high concentrations in river (i.e. 102.7, 56.1 and 4.7 μg/kg) (Pei et al., 2006).

1.2. Fate and degradation mechanism of antibiotics in the environment

Antibiotics are only partially metabolized in an animal's body, in which major fraction excreted in animal feces and urine ultimately reaches water and soil environments through manure or sewer systems (Hamscher et al., 2002; Martínez-Carballo et al., 2007). Use of sewage effluent or sludge in certain agricultural fields (e.g., at public parks and gardening) as well as manure fertilizer for crops production may have serious environmental effects (Sun et al., 2016). Release of antibiotics and antibiotic resistant bacteria (ARB) from manure application can contaminate soils and adjacent water bodies (Harnisz et al., 2015). Although detected concentrations of antibiotics in soils, fertilizers, and aquatic organisms are as low as ppb (parts per billion) level, it may still contribute to the emergence of antibiotic resistant pathogenic bacterial strains in environment (Munir and Xagoraraki, 2007; Pei et al., 2006).

1.3. The development of antibiotic resistant gene (ARG) in bacteria

Presence of antibiotics in the soil environment, even at trace level, is sufficient to exert selection pressure on microbial community that induces transfer of resistant plasmids (Khachatourians, 1998; Thiele-Bruhn, 2003). The ARB passes on their genetic elements through horizontal transfer between pathogens, non-pathogens, and across even distantly related organisms (e.g., between Gram-positive and Gram-negative bacteria) in soil or aqueous environments (Hamscher et al., 2002). Most of the ARBs in aquatic environments have gene structures similar to those of pathogenic bacteria (Oh and Park, 2009). ARBs can have an adverse effect on human health as the pathway for ARB infection in humans originates from antibiotics contaminated manure. Prevalence of antibiotic-resistant pathogens in ecosystem has been a growing problem around the world (Pruden et al., 2006).

TC, OTC, and STZ are the most commonly used veterinary antibiotics and are non-degradable in aquatic environment (Jeong et al., 2010; Leston et al., 2014). The aims of this study were to analyze residual antibiotics concentration profile in cow manure amended soil and its correlation with ARB microbial ecology. For the evaluation of ARB, tests for 16S rDNA PCR and resistance evaluation were performed simultaneously. In addition, it was to determine whether the bacteria might grow using antibiotics as a carbon source. This study will provide information regarding the risk resulting from ARB by examining bacterial resistance against residual antibiotics present in agricultural soil.

2. Materials and methods

2.1. Sample preparation

Cattle manure impacted soil samples were collected in polyethylene bags from depths of 0, 7, and 15 cm below the ground surface from an orchard in Incheon. The samples were transported to the laboratory where experiments and analyses were carried out immediately.

2.2. Antibiotic extraction methods

In order to extract TC and OTC from the soil and manure samples, 0.1 M EDTA-McIlvaine buffer was used as a solvent. The buffer was prepared by dissolving 2.10 g monohydrate citric acid, 2.834 g disodium hydrogen phosphate, and 3.72 g EDTA in 100 mL of deionized water. After that, the pH was adjusted to a final value of 4 (Brandsteterova et al., 1997). In the case of STZ, EDTA-McIlvaine buffer (pH 6) with methanol (10:90) was used as extraction solvent (Martínez-Carballo et al., 2007). A portion of lyophilized soil sample (60 g) was extracted with EDTA-McIlvaine buffer while incubated at 150 rpm for 20 min, ultrasonically treated (Sonictopia, STH-500S, Korea) for 10 s, and then filtered with a 0.45 μm membrane filter. Processed samples were further purified using a solid phase extraction (SPE) cartridge (HLB Oasis 60 mg, 3 mL, Waters, Milford, MA) in 8 mL of methanol (Table 1, Fig. 1). The sample was concentrated finally to 1 mL under nitrogen gas purging evaporator system (Turbovap 2, Zymark Co., Hopkinton, MA).

2.3. Cultivation of ARB and multi-drug resistant bacteria (MRB)

For ARB cultivation, each of the antibiotics was added to R2A agar for isolating general heterotrophic bacteria (Oh and Park, 2009) or to soybean-casein digest agar (TSA) for identifying general culture. The concentrations of antibiotics used were 16 mg/L (OTC), 30 mg/L (TC), and 200 mg/L (STZ) (Harnisz et al., 2015). For MRB cultivation, TC, OTC, and STZ were injected individually and in combination into R2A or TSA medium on which microorganisms had been isolated from a soil sample referring to that the environmental sources in nature are exposed to several antibiotics. The soil samples had been previously prepared by suspending them (20 min) in saline water and then centrifuging for 20 min at 3000 rpm (HA-1000-3, Hanil Science, Daejeon, Korea). Depending on the medium used, 1,000 to 10,000 times diluted ARB and MRB suspensions (0.1 mL) were spread onto each of the antibiotic-amended plates (Brooks et al., 2007). Thereafter, the microorganisms were incubated at 30 °C (VS-1203P 1N, Vision science, Seoul, Korea) for 24 h; after which the colonies on the agar plates were counted manually. The population of resistant bacteria was determined as an average of the replicates.

2.4. Identification of ARB and MRB by 16S rDNA

2.4.1. Extraction of total genomic DNA

The bacterial colonies incubated on the agar plates were suspended in 1 mL of R2A or TSA liquid medium and incubated at 30 °C (VS-1203P 1N, Vision Science, Seoul, Korea) for 24 h. After centrifugation at 13,000 rpm for 5 min, total genomic DNA extraction was performed on the precipitates. For this, the precipitates were ground by bead beating using a FastPrep® Instrument (Bio101 system, Q-bio Gene, Carlsbad, CA) for 5 s at Speed 4, with a FastDNA® SPIN Kit (MP Biomedicals, Santa Ana, CA). The prepared sample was then stored at −20 °C (Park et al., 2008).

2.4.2. Analysis of 16S rDNA

The bacterial isolates were identified using 16S rDNA gene

Table 1
The steps for the solid phase extraction (SPE).

Steps	Solvent	Loading volume	Others
Conditioning	Methanol	6 mL	
	DI Water	6 mL	
Sample loading	Sample	50 mL	5 mL/min
Washing	DI Water	6 mL (2 times)	
Drying			15 min
Elution	Methanol	8 mL	
Evaporation		1 mL	