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Research article

Fate of tetracycline resistance in synthetic livestock carcass leachate for two years

Dennis Espineli Salcedo [a], Sungpyo Kim [a, b, *]

[a] Program in Environmental Technology and Policy, Korea University, Sejong, 30019, Republic of Korea
[b] Department of Environmental Engineering, College of Science and Technology, Korea University, Sejong, 30019, Republic of Korea

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ABSTRACT

To simulate the fate of antibiotic resistance in leachate from anaerobic carcass landfill site, anaerobic reactors were set-up and their antibiotic resistance activities were monitored for 2 years. Initially, *Escherichia coli* DH5α with tetracycline resistance pB10 plasmid was inoculated in nutrient rich anaerobic reactors. The fate of tetracycline resistant bacteria (TRB) was tracked by analysis using culture-based method, EC_{50} (half maximal effective concentration), and quantitative polymerase chain reaction (qPCR). Based on the temporal pattern of EC_{50} during the study period, TRB continuously increased during Phase I (0–250th day), went down in Phase II (after 250th day to 500th day), and then increased again by the end of Phase III (after 500th day to the 774th day). Interestingly, pB10 plasmid accumulated in the system as the community diversities increased over time. At the end of experiment, the tetracycline resistance microbial communities were investigated by 16s RNA gene-based pyro sequencing. The results of this study indicated that leachate with high organic strength in anaerobic conditions could be an antibiotic resistant point source in several year periods.

1. Introduction

The massive outbreak of foot-and-mouth disease (FMD) occurred in South Korea from late 2010 to 2011, which lead to the massive slaughter of hundreds of thousands of pigs in order to contain it (Kim and Kim, 2012). One major problem that the epidemic brought about was the widespread disposal conducted within the landfill leachate. The treatment of high organic and nutrient strength of carcass leachate is a challenging task for environmental engineers. Studies showed the negative impact of carcass burial on nearby groundwater and the water quality (Choi et al., 2013; Yuan et al., 2013). The treatment of potentially hazardous and regulated constituents of a landfill leachate is of great interest to researchers (De Sotto et al., 2016; Li and Champagne, 2009).

Of a particular concern, included in this case, were the suspected leachate antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) spread in the environment. It has been reported previously that the antibiotic use in Korea is much higher than in the Organization for Economic Cooperation and Development (OECD) countries, and contaminates the aquatic environment (Lee et al., 2008; Park et al., 2005). Significant amounts of antibiotics and antimicrobial compounds are used for livestock to prevent epidemic disease or for growth promoting purposes. In addition, only 10–20% of antibiotics react in the animal body, and the rest are excreted into the environment in animal manure (Kim et al., 2010; Park et al., 2007). Although previous studies showed that antibiotic residues could disappear during manure storage (Kemper et al., 2008), the genes that cause antibiotic resistance can still be present and that increases the risk of antibiotic residues spreading throughout the environment. Other researches also showed that more abundant ARB and ARGs are found in animal guts, or feces which are dosed with antibiotics rather than those raised without antibiotic administration (Lim et al., 2007; Zutic et al., 2013). ARB were also isolated from bovine and goat milk (Chung et al., 2009; Kwon et al., 2005), and meat specimens (Lee, 2003). It was also found that higher diversity and abundance of ARB were found in domesticated animals in Korea (Unno et al., 2010). Accordingly, the problem might exist in conjunction with the proliferation and propagation of genetic material in carcass sites which might contain antibiotics and enteric based antibiotic resistance. After carcass sites were set-up, carcass leachate can be produced from

* Corresponding author. Science and Technology Building, Korea University Sejong Campus, Room 509, 2511 Sejong-ro, Sejong City, 30019, Republic of Korea.
E-mail address: ub1905ub@korea.ac.kr (S. Kim).

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the site and the leachate can accelerate the development of ARB and ARGs and transportation (Wu et al., 2015; Zhang et al., 2009).

At the same time, the physicochemical properties of leachate from livestock carcass sites can change since livestock ferment under anaerobic condition (Threedeach et al., 2012), and this result to hydro geochemical and microbial characteristic changes (Kaown et al., 2014). Other studies attempted to calculate the greenhouse gas emissions, even observed prior pH changes and chemical oxygen demand (COD) removal from animal carcass burial (Yuan et al., 2012), and organic component changes in leachate (Kwon et al., 2014). Therefore, enteric dominated ARB communities could shift under unsterilized carcass conditions since the fermentation of substrate can result in the changes of bacterial communities (Yang et al., 2015).

Carcass landfill leachate can contain antibiotics used to treat diseases in livestock. Tetracycline, one of the most widely used antibiotics both by humans and veterinary purposes (Bailey et al., 2016; Blasco et al., 2009; Kemper et al., 2008; Oliver et al., 2011), has been detected in the environment mostly near carcass burial sites, in livestock wastewater or in carcass leachate; and thus needs much attention to eradicate (Gibs et al., 2013; Li, 2014; Polubesova et al., 2006). Tetracycline inhibits bacterial protein synthesis by preventing aminoacyl-tRNA from binding to the bacterial ribosome (Roberts, 1996). Some studies have investigated that there is incomplete removal of pharmaceutical compounds from the environment due to low efficiency of the treatments (Bueno et al., 2012). The low concentrations of antibiotics can increase the development of antibiotic resistance and the transfer of ARGs (Kim et al., 2014; Oh et al., 2014; Salcedo et al., 2014).

Most of previous studies, a single antibiotic concentration in media is used for evaluating antibiotic resistant bacteria abundance. However, this method provides limited information on the distribution of antibiotic resistant bacteria in environment (Kim et al., 2007a). This drawback can be compensated by using multiple antibiotic concentrations (Harwood et al., 2000; Kim et al., 2007a, 2007b; Whitlock et al., 2002).

Although several researchers have written about the fate of microbial and chemical contaminants of groundwater around livestock mortality burial sites (Kim and Kim, 2012; Yang et al., 2015), until now, little has been documented regarding the fate of antibiotic resistance as a function of the characteristics of leachates in carcass landfills, which is believed to be under the anaerobic condition, with no further substrate addition under the long term monitoring manner. In particular, tetracycline is widely used in livestock, and is of much interest to many researchers due to its effects to the development of antibiotic resistance (Chopra and Roberts, 2001; De Sotto et al., 2016; Kim et al., 2007b, 2014), especially *E. coli* in landfills (Threedeach et al., 2012).

Therefore, this study was designed to track the fates of TRB and the tetracycline resistance plasmid in the livestock carcass simulated leachate under anaerobic batch reactor in a long-term period using culture-based method, EC_{50}, and the quantitative polymerase chain reaction (qPCR). EC_{50} is calculated by multiple antibiotic concentrations for compensating the drawback of TRB evaluation by single tetracycline concentration. As far as authors know, this work is the first systemic approach to understand the fate of antibiotic resistance in a carcass leachate.

2. Materials and methods

2.1. Preparation of microorganisms

E. coli DH5α (pB10) was selected as model enteric tetracycline resistant strain in our study. There are several reasons to select this strain as a model. First, the pB10 plasmid was isolated from a wastewater treatment plant, has tetracycline resistance property, and the complete nucleotide sequence was identified (Schluter et al., 2003). In addition, it was confirmed that pB10 plasmid was found in some carcass leachates in Korea (Fig. 1, data is not published), is transferable among microorganisms, and has potential risk in the environment (Kim et al., 2014).

E. coli DH5α (pB10) was first grown in liquid medium in Erlenmeyer flasks. One colony was transferred from the 24-h solid culture into 200 mL lysogeny broth (LB). The strain was incubated for 12 h under orbital agitation (Vision Scientific Co., Ltd., KMC-8480S) at 150 rpm at 37 °C, to obtain a planktonic culture on the early exponential growth phase. After incubation period, optical densities (OD) were checked using spectrophotometer (Bio-Rad SmartSpec Plus Spectrophotometer), approximately 5 AU taken at 600 nm wavelength. The bacterial suspension was used as the inoculum at a concentration of 10^8 colony-forming units (CFU) mL^{-1}.

2.2. Synthetic carcass landfill leachate

2.2.1. Physicochemical characteristics of the synthetic leachate

A synthetic carcass leachate was prepared and derived from other references (Glanville, 1993; MacArthur and Milne, 2002; Pratt, 2009). In addition, real carcass landfill leachate samples were collected from pig and duck burial sites in Cheonan, Republic of Korea in 2011. Total nitrogen (TN), total phosphate (TP) and COD were analyzed as reference to the initial values of synthetic landfill leachate (Table 1).

2.2.2. Synthetic carcass landfill leachate model set-up

Synthetic carcass leachate was filled up in three acrylic glass cylinders. In each cylinder, 5 L synthetic carcass leachate, having about 10^5 CFU of *E. coli* DH5α (pB10) and 2 mg L^{-1} tetracycline were initially added. In maintaining anaerobic condition, N_2 gas was injected for 1 h daily. Dissolved oxygen (DO) concentrations in each reactor was checked regularly and it was maintained at <2 mg L^{-1} during the study period. Liquid agitation of the contents using N_2 gas was achieved through a Fisher-direct drive stirrer (Fisher Scientific Co., Pittsburg, PA). Sampling of a composite of 75 mL was done every 15 days. The outlet was first disinfected and then, 25 mL sample was withdrawn from each of the reactors collected in a sterilized 250 mL HDPE wide-mouth Nalgene bottle (Thermo Scientific). Samples were tested for physicochemical properties. Deoxyribonucleic acid (DNA) was extracted from 50 mL of the sample. Fig. 2 shows the three reactors at day 1 (Fig. 2a) and day 352 (Fig. 2b).

2.3. Physicochemical tests

DO concentrations were measured three times a week during the aeration time using Orion 5 Star (Thermo Scientific) to maintain the anaerobic condition. pH was monitored using an Orion 3 Star pH portable meter (Thermo Scientific) at room temperature (APHA, 2005). COD was measured by adding 2 mL of each sample into Hach Digestion Solution for COD following the manufacturer's procedure. Digestion was done using a DRB200 Reactor at 150 °C for 120 min. Samples were then cooled down to room temperature and COD demand in mg L^{-1} was analyzed using a Hach DR/2010 Portable Data logging Spectrophotometer (Jirka and Carter, 1975; USEPA, 1980). TN and TP concentrations were measured using a Total Nitrogen Reagent Set and a Total Phosphate Reagent Set (Hach, USA), respectively. Samples were also diluted to 2×10^{-2} first. Digestion was also done using a DRB200 Reactor. Furthermore, the vials were removed from the reactor and cooled down to room temperature. Finally, the samples were read by a UV/vis