High-throughput nucleotide sequence analysis of diverse bacterial communities in leachates of decomposing pig carcasses

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Abstract

The leachate generated by the decomposition of animal carcass has been implicated as an environmental contaminant surrounding the burial site. High-throughput nucleotide sequencing was conducted to investigate the bacterial communities in leachates from the decomposition of pig carcasses. We acquired 51,230 reads from six different samples (1, 2, 3, 4, 6 and 14 week-old carcasses) and found that sequences representing the phylum Firmicutes predominated. The diversity of bacterial 16S rRNA gene sequences in the leachate was the highest at 6 weeks, in contrast to those at 2 and 14 weeks. The relative abundance of Firmicutes was reduced, while the proportion of Bacteroidetes and Proteobacteria increased from 3-6 weeks. The representation of phyla was restored after 14 weeks. However, the community structures between the samples taken at 1-2 and 14 weeks differed at the bacterial classification level. The trend in pH was similar to the changes seen in bacterial communities, indicating that the pH of the leachate could be related to the shift in the microbial community. The results indicate that the composition of bacterial communities in leachates of decomposing pig carcasses shifted continuously during the study period and might be influenced by the burial site.

Keywords: pig, decomposition, leachate, bacterial community, pyrosequencing.

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Introduction

Foot and mouth disease (FMD) is highly contagious and is caused by members of the genus Aphthovirus, represented by various serotypes, such as A, O, C, SAT-1, SAT-2, SAT-3, and Asia-1 (Brooksby, 1982; Seellers, 1995).

FMD is especially fatal in young animals as a result of myocardial damage, while the mortality rate of FMD might be low in adult animals (Sharma and Das, 1984; Woodbury, 1995; Domingo et al., 1990; Gruman and Baxt, 2004). Therefore, FMD-infected animals are slaughtered immediately and buried according to guidance on animal carcass mass burial procedures developed by each country. Despite an extensive nationwide quarantine effort, South Korea experienced the worst FMD epidemic in the country’s history during 2010-2011, resulting in the culling of more than 3.5 million heads of Artiodactyla, including cattle, goat and pigs (Ahn, 2012).

Although burial is the most common method used to dispose of large numbers of deceased livestock, the potential pathogenic content of the carcass associated health problems to residents living near the burial site have resulted in widespread public health concerns (NRC, 2002). Moreover, leachates derived from the decomposition of animal carcasses consist of organic intermediates, volatile chemicals, and saprotrophic organisms, which may pose a high contamination risk to soil and groundwater when leaking through the basement layer (Christensen et al., 1994; Röling et al., 2001; Tian et al., 2005).

The rate at which animal carcasses decompose in the burial pit can be affected by temperature, humidity, the depth of the pit, chemical composition, and other environmental factors (Mann et al., 1990; McDaniel, 1991; Vass et al., 1992; Paul and Clark, 1996; Gill-King, 1997; Carter and Tibbett, 2006; Pratt et al., 2010). Generally, temperature and pH can accelerate microbial decomposition by changing the soil microhabitat (Paul and Clark, 1996; Blagodatskaya and Anderson, 1998). For example, the metabolic activity of a mesophilic microbial population...
increased approximately two-fold with an increase in temperature from 10 °C to 30-35 °C (Van’t Hoff, 1898; Conant et al., 2004). Moreover, pH has a pivotal role in the type of microorganisms that predominate in different soils (Lynch and Hobbie, 1988; Matthies et al., 1997). Other environmental factors, including moisture, dissolved oxygen, conductivity and the like also play an important role in the microbial community composition (Keener et al., 2000; Pettersson M, 2004, Doctoral thesis, Lund University, Sweden). Soil properties may also affect the microbial community (Carter et al., 2007) and, hence, the carcass decomposition process (Pfeiffer et al., 1998).

In a forensic study, the bodies of obese people decomposed faster than those of average weight individuals due to the greater amount of liquid present in the tissues, which contributes to the spread and proliferation of bacteria (Campobasso et al., 2001). The high organic load of the leachate could cause drastic changes in aquifer geochemistry and microbiology downstream of landfills (Roling et al., 2001), and leachates originating from the decomposition of buried pig carcasses, as investigated here, could cause similar changes in soil and in the geochemical characteristics of water as well.

The methods for burying dead livestock or the characteristics of uncultured microbial communities in the burial site have not been studied in detail. The presence of many uncultured bacteria has been confirmed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and phospholipid fatty acid (PLFA) profiling. However, such studies primarily focused on specific microbial communities in landfill leachate plumes (Ludvigsen et al., 1997; Sasaki et al., 2009; Yang et al., 2012). The advent of next generation sequencing techniques, such as bar-coded pyrosequencing, can elucidate microbial community structures at a much higher resolution than previously possible. Moreover, the large dataset produced by pyrosequencing enables the detection of rare microbes and to perform phylogenetic comparisons of microbes living in a particular environment (Liu et al., 2007, 2008; Hamady et al., 2010; Kirchman et al., 2010). Therefore, the goal of the present study was to take advantage of pyrosequencing to investigate the identities of members of microbial communities in leachates originating from the decomposition of pig carcasses over a period of 98 days.

Materials and Methods

Testing device

A lab-scale system was designed based on animal carcass mass burial procedures developed by the Korean Ministry for Food, Agriculture, Forestry and Fisheries. The lab-scale system, (1/7 normal size), was 0.54 m (l) x 1.4 m (w) x 0.85 m (h), approximately 0.67 m$^3$ in volume, and is described in detail by Yang et al. (2012). Three lab-scale systems, each of which can accommodate an approximately 115 kg single pig carcass, were placed in a room at 35 °C. The leachate samples were collected using a peristaltic pump from each of three containers once each week for the first month and then at 6 and 14 weeks. Serial collected sample sets from the triplicate system during decomposition were used for molecular analysis. The pH was measured immediately after collection using a bench-top pH meter (Orion 4 STAR, Thermo Scientific Inc., Beverly, USA), and then the leachate samples were stored at -80 °C.

PCR amplification and pyrosequencing

Total genomic DNA was extracted from leachates using a Fast DNA Spin kit for soil (MP Bio, Texas, USA). Fragments of the V1-V3 regions of the 16S rDNA were amplified using fusion primers from genomic DNA extracted from leachate samples (Hur et al., 2011). Each PCR reaction was performed in a final volume of 50 μl with 5 μl of 10X Taq buffer, dNTP mixture (Takara, Shiga, Japan), 10 μM of each primer and 2 U of Taq polymerase (Ex Taq, Takara) using a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA, USA). Amplification reactions were performed as follows: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 7 min. After visualizing the PCR product using gel electrophoresis and the Gel Doc system (Bio-Rad), the amplicons were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Purified amplicons from different samples (same concentration of each sample) were pooled and purified using an AMPure bead kit (Agencourt Bioscience, Beverly, MA, USA). Pooled products were amplified on the sequencing beads using emulsion PCR, and the beads were deposited on a 454 Picotiter Plate and sequenced using a GS Junior system (Roche, Branford, CT, USA) according to the manufacturer’s instructions.

Analysis of pyrosequencing

Pyrosequencing analysis was performed according to published methods (Chun et al., 2010; Hur et al., 2011; Kim et al., 2012a). The raw data obtained from different samples were sorted by their unique barcodes. We discarded sequences containing two or more ambiguous nucleotides (N), low quality score (average score < 25), or reads shorter than 300 base pairs (bp). After deleting chimeric sequences, the taxonomic assignment of each read was conducted using the EzTaxon-e database, which contains representative phylogenotypes of cultured and uncultured sequences in the GenBank database. The phylogenetic positions were manually edited (Kim et al., 2012b). The richness and diversity of samples were calculated according to the Chao1 estimation and Shannon index at the 3% dissimilarity level using the Mothur program (Schloss et al., 2009). To compare different read numbers among samples, random subsampling was conducted using CLOri-community software (Chunlab, Inc., Seoul, Korea). The phylogenetic
distances between communities were estimated using Fast UniFrac (Hamady et al., 2010) and visualized with principal coordinate analysis (PCoA). The significances of the differences between community structures were calculated using Libshuff analysis (Singleton et al., 2001). Pyrosequences obtained in this study were uploaded to the EMBL SRA database under the study accession number ERP002343 (http://www.ebi.ac.uk/ena/data/view/ERP002343).

Results and Discussion

The analyses of bacterial communities in leachates from different stages of the decomposition of pig carcasses were conducted by pyrosequencing of 16S rRNA gene amplicons. A total of 51,230 reads were obtained from six different sampling times, and normalized read numbers in each sample were used to compare estimated values among samples (Table 1). The minimum value of Good’s coverage was 81% (week 6 sample), and the maximum value was 90% (week 2 sample). The highest and lowest numbers of operational taxonomic units (OTUs) was observed at week 6 (1,002 OTUs) and 2 (549), respectively. The diversity of community composition changed during the periods of decomposition. The Shannon index decreased from 1 (4.10) to week 4 (3.22), increased at week 6 (4.58), and decreased again at week 14 (3.06). These results indicate that the bacterial communities in leachates at 1 and 6 weeks consisted of various bacterial species and were complex. The presence of dominant bacterial species decreased the diversity of the bacterial community observed during the early stage.

The rarefaction curves of the samples are presented in Figure 1. The highest richness of the bacterial community was detected in the 6-week sample and the lowest at 2 and 14 weeks. The dominant phylum was Firmicutes in all samples, and the abundance of Proteobacteria and Bacteroidetes was increased in the 6-week sample (Figure 2). The shift in the composition of the bacterial community was investigated in more detail at the genus level. The abundance of unclassified bacteria within Tissierella genus increased from weeks 2 to 4 (27% of total reads at week 1, > 46.1% from weeks 2 to 4). Peptostreptococcus increased at 14 weeks (38.7% of total reads). Ruminal Peptostreptococcus

Table 1 - Comparison of pH to the abundance of bacterial 16S rDNA sequences of leachates from decomposing pig carcasses.

| Sample (week) | pH   | Analyzed reads | Normalized reads | Average length (bp) | Observed OTUs | Estimated OTUs (Chao1) | Shannon Index | Good’s coverage |
|---------------|------|----------------|------------------|---------------------|---------------|------------------------|---------------|-----------------|
| 1             | 6.59 | 4,100          | 4,100            | 454.44              | 667           | 2,910.31               | 4.10          | 0.87            |
| 2             | 6.47 | 5,230          | 4,100            | 457.63              | 549           | 1,977.74               | 3.05          | 0.90            |
| 3             | 6.46 | 17,035         | 4,100            | 455.34              | 799           | 5,147.62               | 3.45          | 0.84            |
| 4             | 6.51 | 7,805          | 4,100            | 454.88              | 650           | 3,177.83               | 3.22          | 0.87            |
| 6             | 8.79 | 5,674          | 4,100            | 458.89              | 1,002         | 4,349.40               | 4.58          | 0.81            |
| 14            | 6.48 | 11,386         | 4,100            | 455.65              | 593           | 2,645.86               | 3.06          | 0.88            |

Figure 1 - Rarefaction curves of six different samples obtained from leachates. Each number indicates the time of sampling.
plays an important role in the degradation of valine, isoleucine, and leucine (Chen and Russell, 1989). The relative abundance of *Anaerosalibacter* was the highest at week 1 (over 11% of total reads); this genus was isolated from oil sludge and is reported to be a reducing species during the decomposition process (Rezgui et al., 2012).

*Tissierella* and *Tepidimicrobium* are anaerobic species that reduce protein and amino acids, and these bacteria generally increased during decomposition (Harms et al., 1998; Slobodkin et al., 2006). Our present data reveal that there was a gradual reduction of *Clostridium* spp. and *Anaerosalibacter* from weeks 2 to 14 while uncultured *Tissierella* spp. (AB175363 g and EF559164 g) and *Tepidimicrobium* was increased at week 6. The relative abundance of the various genera increased at week 6, and this produced the highest diversity of bacteria. After six weeks the leachate might have contained a variety of organic compounds that supported the growth of diverse classes of bacteria.

The result of the microbial communities grouped by UniFrac and PCoA is shown in Figure 3. The community compositions in the two and four weeks samples were similar, while the compositions changed after six and 14 weeks, which suggests that different bacteria were active at each stage of decomposition. Furthermore, the first principal coordinate (PC1, variance of 30.0%) separated the microbial communities in accordance with the pattern of pH suggesting that major contributor to affect dynamics of the microbial communities might be pH, although other factors affecting microbial community did not be shown in this study.

Although most sequences were identified as uncultured bacteria at species, the assignment using the Eztaxon-e database provided detailed information about the uncultured bacteria (Figure 4). Uncultured bacteria

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**Figure 2** - Double pie charts of bacterial communities. The inner and outer pies represent the composition of phyla and genera, respectively. The numbers above each pie indicates the sampling time. The nomenclature of phylotypes is based on the EzTaxon-e database (Kim et al., 2012b; http://eztaxon-e.ezbiocloud.net/).
similar to *Tissierella* (*Tissierella_f_uc_s*) were the most dominant species among all samples, and most of the sequences identified as uncultured *Tissierella* were similar to GenBank accession numbers AB175363 and EF559164. Uncultured *Tissierella* AB175363 is a mesophilic anaerobic protein-degrading bacterium, and uncultured *Tissierella* EF559164 is involved in the methanization of cellulose under mesophilic conditions (Tang et al., 2005; Li et al., 2009). Therefore, uncultured *Tissierella* detected here may play a main role in the degradation of pig proteins during 14 weeks. The numbers of *P. russelli* was increased significantly at week 14, and *Clostridium* spp. were abundant in the week 1 sample. *P. russelli* was isolated from a swine-manure storage pit, and the presence of this species could be related to the decomposition stage of organic materials (Whitehead et al., 2011). *Clostridium* spp. are ubiquitous anaerobes found in various environments and are potentially pathogenic. *Clostridium* spp. were isolated from the gastrointestinal tract of pigs, and are abundant at relatively early stages of decomposition (Varel et al., 1995; Alvarez-Perez et al., 2009; Garcia et al., 2009). Although most of the bacteria detected are anaerobic, the proportion of the aerobic bacterium *Comamonas kerstersii* was increased at week 6. Species of *Comamonas* are also present during anaerobic acidification of pig excreta (Zhang et al., 2011). The diverse uncultured bacteria detected in this study provide further information about leachate decomposition in the environment.

Recently it was reported that limestone-containing hydrated lime and quicklime affected the decomposition of pig carcasses by changing the pH in burial pits (Schotsmans et al., 2012). Quicklime was used in the present study to decompose the pig carcasses, which might have contributed to the sharp increase in pH to 8.79 caused by permeation of the leachate through the layer of quicklime during six weeks (Table 1). This increased pH might be related to the highest level of diversity in this sample. The shifts in bacterial communities indicate that different bacterial species played a role during specific decomposition periods and generated and degraded organic materials that differed during each period.

Investigations of shifts in the population of bacterial communities are important, because the leachate affects the conditions of adjacent land and the surrounding environment. Thus, microorganism can influence the ecosystem balance through the activities of their metabolites (Stahl
and Tiedje, 2002). Furthermore, the decomposition of carcasses adversely affects the environment for as long as two to ten weeks after burial (McDaniel, 1991). Our present findings indicate a continual shift in the composition of bacterial communities in leachates generated during the decomposition of pig carcasses. The bacterial community in the leachates could affect the surrounding environment, including soil and groundwater at or near the burial site, potentially leading to changes in the composition of organic materials, indigenous microbes, and symbionts. These changes can be expected to adversely affect public health.

In conclusion, we found shifts in bacterial communities in leachates of decomposing pig carcasses during 14 weeks. Different bacterial groups dominated and may play major roles in the decomposition during specific time intervals. Uncultured *Tissiellera* spp. and *Peptostreptococcus* spp. were present in abundance, and the pH was an interactive factor in the decomposition of pig carcasses. The results illustrate the population dynamics of bacterial communities and revealed the predominant bacterial species in such leachates. This information holds promise to improve and implement measures against any future outbreaks of FMD in Korea and other countries and could also be helpful for maintaining the health of ecosystems. Clearly, further studies of burial sites areas will be required to manage and to avert adverse effects on public health.

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Internet Resources

EzTaxon-e database, http://eztaxon-e.ezbiocloud.net (accessed at 2012.10.02).

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