Characterization of Microbial Community in the Leachate Associated with the Decomposition of Entombed Pigs

Yang, Seung-Hak1†, Sun Hwa Hong2†, Sung Back Cho1, Joung Soo Lim2, Sung Eun Bae3, Heekwon Ahn4, and Eun Young Lee2,*

1Animal Environment Division, National Institute of Animal Science, R.D.A., Gyeonggi 441-706, Korea
2Department of Environmental and Energy Engineering, Suwon University, Gyeonggi 445-743, Korea
3Women’s Health, School of Medicine, Hodgkin Building, King’s College London, London SE1 1UL, United Kingdom
4Department of Animal Biosystems Science, Chungnam National University, Daejeon 305-764, Korea

Received: May 4, 2012 / Revised: June 19, 2012 / Accepted: June 20, 2012

Foot and mouth disease (FMD) is one of the acute infectious diseases in hoofed and even-toed mammals, including pigs, and it occurs via acute infection by Aphthovirus. When FMD is suspected, animals around the location of origin are typically slaughtered and buried. Other methods such as rendering, composting, and incineration have not been verified in practice in Korea. After the FMD incident, the regular monitoring of the microbial community is required, as microorganisms greatly modify the characteristics of the ecosystem in which they live. This is the result of their metabolic activities causing chemical changes to take place in the surrounding environment. In this study, we investigated changes in the microbial community during a 24 week period with DNA extracts from leachate, formed by the decomposition of buried pigs at a laboratory test site, using denaturing gradient gel electrophoresis (DGGE) with a genomic DNA. Our results revealed that Bacteroides coprospuis, which is common in pig excreta, and Sporanaerobacter acetigenes, which is a sulfur-reduced microbe, were continuously observed. During the early stages (0~2 weeks) of tissue decomposition, Clostridium cochlearium, Fusobacterium ulcerans, and Fusobacterium sp., which are involved in skin decomposition, were also observed. In addition, various microbes such as Turicibacter sanguinis, Clostridium haemolyticum, Bacteroides propionicifaciens, and Comamonas sp. were seen during the later stages (16~24 weeks). In particular, the number of existing microbial species gradually increased during the early stages, including the exponential phase, decreased during the middle stages, and then increased again during the later stages. Therefore, these results indicate that the decomposition of pigs continues for a long period of time and leachate is created continuously during this process. It is known that leachate can easily flow into the neighboring environment, so a long-term management plan is needed in burial locations for FMD-infected animals.

Keywords: Denaturing gradient gel electrophoresis (DGGE), foot and mouth disease (FMD), leachate, microbial community

Foot and mouth disease (FMD) is an acute infectious disease caused by Aphthovirus, which belongs to the family Picornaviridae. The foot and mouth disease virus (FMDV) is a prototypical member of the genus Aphthovirus. There are seven FMDV serotypes; A, O, C, SAT-1, SAT-2, SAT-3, and Asia-1 [20]. FMD is highly contagious and can infect most types of Artiodactyla, including cattle, sheep, pigs, and even wild animals such as water buffalo [4]. The mortality rate of FMD is less than 5%, but it reduces the value of farm animals owing to consequential growth and movement disorders. In addition, it has been reported that whereas adult animals typically may die as a result of the infection, young animals normally die as a result of myocardial damage [6, 21, 26]. There are several factors that make FMD a real and common threat to farmers and a national food supply. The primary factors can be described as FMD’s high contagiousness, wide geographical distribution, broad host range, ability to establish carrier status, antigenic diversity leading to poor cross-immunity, and the relatively short duration of immunity. It has been noted that, because of these factors, facilities and programs are insufficient to adequately control an outbreak of FMD [22]. Therefore, when FMD is suspected, animals around the suspected locations have movement restrictions imposed and animals in the place of origin are slaughtered.
and buried. These methods are utilized in the absence of effective treatment options.

Large amounts of leachate and seriously unpleasant odors are often generated in the burial areas by decomposing FMD-infected animals. Leachate starts to be produced approximately one week after burial. Leachate contains a large number of organisms, and contaminates soil and groundwater through the basement layer [23]. Contaminated soil and underground water make for a serious environmental issue with potential risks for the immediately local ecosystem as well as for neighboring areas. A large number of animal burials took place following a serious outbreak of FMD in Korea in November 2010. Serious problems resulting from leachate have not yet been reported; however, it would be prudent to prepare for the possibility that contamination has already occurred and may continue to occur from these, or future, burials.

The regular environmental monitoring of burial areas importantly includes the monitoring of microbe changes according to their correlation with pollution sources [18]. Recently, various research techniques to study the microbial community, but not the individual microbe, have been developed in the microbial ecosystem [14, 17, 25]. Amongst these, molecular biological methods are known to be accurate, rapid, and well documented by previous researchers [1, 8, 9, 11, 13–17, 24, 27]. In particular, denaturing gradient gel electrophoresis (DGGE), using urea or formamide as a denaturant, is a relatively simple method and it can compare microbes numerically and quantitatively.

Therefore, in this study, we regularly monitored microbes using the DGGE method, with genomic DNA from leachate formed by the decomposition of entombed pigs at a laboratory test site mimicking methods used for pig slaughter and burial after FMD discovery, in order to better understand features of the microbial community in the field.

**MATERIALS AND METHODS**

**A Designed Lab-Scale Experiment**

A laboratory pig carcass decomposition testing site was designed based on animal mortality mass burial protocols developed by the Korean Ministry for Food, Agriculture, Forestry and Fisheries. In order to mimic the decomposition of pig carcasses at real burial sites, lab-scale systems scaled down to about 1/7th normal size were used (Fig. 1). The area volume was around 0.67 m³ with a 1.4 m width, 0.54 m length, and 0.85 m height. A pig carcass of around 115 kg in weight was placed on top of a soil and quick lime layer (7.5 cm soil + 0.75 cm quicklime + 7.5 cm soil). This was followed with additional layers of soil and quicklime (7.5 cm soil + 0.75 cm quicklime + 7.5 cm soil). Approximately 120 kg of soil weight was laid on top of the upper layers of the soil and quicklime so as to prevent the carcasses abdomen from swelling and distending. All three replicate containers were subjected to a constant-temperature room at 35°C in order to increase the reaction rate of the decomposition of the pig carcass. Leachate samples were collected from each of the three containers on a weekly basis for a month, and then monthly for five months. The collected leachate samples were analyzed using denaturing gradient gel electrophoresis (DGGE) in order to examine microbial community variations during the decomposition of the pig carcass.

**Experimental Methods**

Total genomic DNA was extracted from the leachate of the containers using a BIO101 kit (MB Bio, USA). Next, a fragment of the variable V3 region of 16S rDNA was amplified by PCR primers 341f-GC (5’-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3’) and 907r (5’-CCC CGT CAA TTC ATT TGA GTT-3’). The PCR reaction was performed in a thermal cycler system (TaKaRa PCR Thermal Cycle, Japan) at 95°C for 5 min, followed by 28 denaturation cycles at 95°C, annealing at 60°C for 30 s, and extension at 72°C for 30 s. A final extension was performed at 72°C for 5 min. DGGE analysis was conducted using a DCode system (Bio-Rad, USA). Samples of PCR product (20 µl) were loaded onto 6% (w/v) polyacrylamide gels in 1× TAE buffer. The polyacrylamide gels were made with a
linear denaturing gradient ranging from 35% denaturant at the top of the gel to 70% denaturant at the bottom. The electrophoresis was run at 50 V and 60°C for 14 h to get a clear image. The gel was analyzed with UVI-band pattern (UVI Soft, UVI-band version 12.14, UK). In addition, typically 19 bands were cut from the DGGE fingerprint to extract DNA and then PCR was conducted with 341f and 907r primers. The full-length sequence of amplified 16S rDNA genes was deposited in the GenBank database of the National Center for Biotechnology Information. The sequence was submitted to the Advanced Basic Local Alignment Search Tool (BLAST) search program to identify the sequences of any closely related organisms. The related sequences were preliminarily aligned with Clustal W, and manually adjusted with the aid of the Clustal X program. Phylogenetic analysis was performed using Bioedit, and the phylogenetic trees were determined using the neighbor-joining method. A phylogenetic tree was constructed with the MEGA4 program [1, 9].

RESULTS AND DISCUSSION

Phylogenetic features are shown in Fig. 2, and species in each period are illustrated in Table 1. A phylogenetic tree indicates whether microbes in the burial area are connected to other groups. Specifically, 19 bands on DGGE gel were cut and then isolated; however, three of the 19 bands were not isolated, with the rest shown in Fig. 2. The most clear band 1 (G1) was Bacteroides coproxis and was close to band 10 (G10) and band 16 (G16) (UPGMA and maximum-parsimony with bootstrap values of 100% and 99%, respectively). Most bands, excluding bands 3 (G3), 4 (G4), and 17 (G17), were placed in similar locations.

The microbes revealed in this study were divided into three groups depending on their existing stages, such as (i) present in all stages, (ii) present only in the early stages, and (iii) present only in a later stage. Our results showed that G1, G2, G4, G10, and G13 were detected in every sample after the event of pig burial. G1 and G10 were very similar to Bacteroides coproxis, separated from the pig excreta hole with a 99% similarity. G2 was similar to Sporanaerobacter acetigenes known as a sulfur-reduced microbe that oxidizes acetate and succinates organic matter. A large amount of acetate (10,000 ppm) and succinate (5,000 ppm) was produced in the leachate during the decomposition, and malodorous substances affiliated with sulfur were formed in animal excreta, so Sporanaerobacter acetigenes production seemed to be continuous. G4 was isolated as an uncultured bacterium. G13 was observed in every sample but too blurred to be isolated on DGGE gel.

G3, G5, G6, and G11 were observed until 2 weeks after burial. The bands were isolated as Acidovorax sp. (G3), Tepidanaerobacter syntrophicus (G5, 6), and Clostridium cochlearium (G11). Acidovorax sp. is a denitrifying bacterium known to convert nitric acid or nitrous acid to nitrogen gas then released in the air [12] and it thrives in an anaerobic environment. An artificial anaerobic environment was maintained inside the reactor, so this could have induced the appearance of Acidovorax sp. In addition, if a large amount of ammonia is generated for 2 weeks during the decomposition, this could also have induced the appearance of nitrification germs in the early stages. In addition, Tepidanaerobacter syntrophicus breaks down into alcohol and lactate under anaerobic conditions [19]. Lactate, succinate, and ethanol are generally generated when organic matter is used as the final electron acceptor.
CHARACTERIZATION OF MICROBIAL COMMUNITY IN THE LEACHATE OF ENTOMBED PIGS

for fermentation. Therefore, *Tepidanaerobacter syntrophicus* seemed to appear in the early stages during the gravest decomposition. *Clostridium cochlearium* is a representative anaerobe of *Clostridium* sp. It is observed in septicemia and able to cause skin necrosis. *Clostridium cochlearium* also appeared in the early stages.

In particular, the G7, G8, and G9 bands were very thick in the 2nd week’s sample, but very dim in the 12th, 16th, and 24th weeks. These were isolated as *Fusobacterium ulcerans* and *Fusobacterium* sp., which are reported to be related to skin necrosis and septicemia [3]. Therefore, these latter two seemed to participate in the decomposition of the skin, which was most active in the 2nd week of burial.

In the later stages (16th to 24th weeks), bands G14, G15, G16, G17, G18, and G19 were observed. G14 ~ G17 were isolated as *Turicibacter sanguinis*, *Clostridium haemolyticum*, *Bacteroides propionicifaciens*, and *Comamonas* sp., respectively, whereas G18 and G19 were not isolated. These are also anaerobes and *Turicibacter sanguinis* is often found in lagoons [16]. *Clostridium haemolyticum* is pathogenic and observed in dying animals. *Bacteroides propionicifaciens* was previously isolated from the straw residues of methane reactions to process farm waste [2] and seemed to appear as a result of the large amount of methane during the decomposition. *Comamonas* sp. can be formed during an anaerobic process in pig excreta [27] and is known to break down acetaldehydes [13]. In addition, *Comamonas* sp. is a denitrifying bacterium [7]. *Acidovorax* sp. seemed to be involved in the early stages of the decomposition, with the nitrogen source considered to originate from protein. *Comamonas* sp. was expected to participate in denitrification in the burial area in the later stages.

The number of microbe species in all samples increased continuously until the 2nd week and then decreased until the 12th week, and then increased again in the 16th to the 24th weeks with new species being observed. *Acinetobacter sp.*, *Arthrobacter cumminsii*, *Bacillus sp.*, *Brevibacterium sp.*, *Pseudomonas aeruginosa*, and *Serratia marcescens* were reported to be possibly present during the decomposition of pigs [11]. However, in the latter results, it was considered that some microbes were derived from excreta (*Bacteroides coprosuis*, *Turicibacter sanguinis*, and *Comamonas* sp.) and some from necrosed skin tissue (*Clostridium cochlearium*, *Fusobacterium ulcerans*, and *Fusobacterium* sp.), whereas others were also related to a foul smell (*Sporanaerobacter acetigenes*, *Acidovorax* sp., *Bacteroides propionicifaciens*, and *Comamonas* sp.). The results in the present paper seem to be different from previous studies. This is because in other studies the microbes were collected from soil, whereas the microbes in this study were collected from leachate. In the case of a human, the body decomposes from the first week after burial with the production of related microbes, followed by gases being generated by the decomposition of the organs expanding for three weeks.

Table 1. Dominant species of leachate bacteria associated with the decomposition of entombed pigs based on 16S rDNA sequences.

<table>
<thead>
<tr>
<th>Band name</th>
<th>Bacteria species</th>
<th>Incubation time (Week, W)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0W</td>
</tr>
<tr>
<td>G1</td>
<td><em>Bacteroides coprosuis</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G2</td>
<td><em>Sporanaerobacter acetigenes</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G3</td>
<td><em>Acidovorax sp.</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G4</td>
<td>Uncultured bacterium</td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G5</td>
<td><em>Tepidanaerobacter syntrophicus</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G6</td>
<td><em>Tepidanaerobacter syntrophicus</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G7</td>
<td><em>Fusobacterium ulcerans</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G8</td>
<td><em>Fusobacterium sp.</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G9</td>
<td><em>Fusobacterium ulcerans</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G10</td>
<td><em>Bacteroides coprosuis</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G11</td>
<td><em>Clostridium cochlearium</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G12</td>
<td><em>Tepidanaerobacter syntrophicus</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G13</td>
<td>NS</td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G14</td>
<td><em>Turicibacter sanguinis</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G15</td>
<td><em>Clostridium haemolyticum</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G16</td>
<td><em>Bacteroides propionicifaciens</em></td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G17</td>
<td><em>Comamonas</em> sp.</td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G18</td>
<td>NS</td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
<tr>
<td>G19</td>
<td>NS</td>
<td>(+) (+) (+) (+) (+) (+) (+) (+) (+)</td>
</tr>
</tbody>
</table>

*NS, not sequenced.*
Human skin tissue and organs are completely decomposed within 50 days. However, a pig has different protein contents and an absence of hair, and therefore, the time scale to complete the decomposition might be different from a human [11]. In addition, environmental factors related to the decomposition can have a drastic effect on the speed of decomposition. Howard et al. [11] investigated microbes during the decomposition of a pig of 47 kg and reported that large amount of microbes were found for 70 days during the experiment. Our results suggest that the regular occurrence of excreta microbes was caused by the relatively slow decomposition due to our pigs’ weight being between 112 and 116 kg.

In conclusion, the specific changes in microbe type and quantity during the decomposition of a vertebrate after burial are not well known from the results of previous studies. Most past studies have reported on the changes in soil microbes during the decomposition of invertebrates [5, 24] rather than vertebrates such as pigs [11]. It has been reported that soil microbes only change slightly owing to weak migration, [9] but leachate is very migratory. Therefore, there is a real possibility that soil and groundwater can be polluted by leachate generated during the decomposition of entombed pigs that have been infected with FMD, or other infectious diseases present in domesticated animals. After a contamination incident, the regular monitoring of the microbial community is required because microorganisms can greatly modify the characteristics of the ecosystem in which they live by causing chemical changes to take place as a result of their metabolic activities. Therefore, our study of the microbial changes in leachate resulting in the immediate burial area is very important. In this study, we built a reaction tank to produce similar field conditions to those seen in the real slaughter and burial of pigs. The microbial community formed during pig decomposition was measured using leachate. A wide variety of microbes were detected during this process, and microbe formation and variation continued for a prolonged period of time, going against our expectations that microbe species would decrease over the period of decomposition of the pigs. As leachate can easily flow into the environment, a continuous long-term management and monitoring system for slaughter and burial sites and surrounding areas after FMD is needed, with DGGE with DNA sequencing using 16S rDNA providing a good practical method for implementation.

Acknowledgment

This work was carried out with the support of the Research Program for Agriculture Science & Technology Development (Project No. PJ907130022012), courtesy of the Rural Development Administration in the Republic of Korea, and the authors would like to express their gratitude for its provision.

References


