보건학석사 학위논문

Association of Microorganisms with Physico-chemical and Microbiological Characters of Ground Water near Foot-and-Mouth Disease Burial Sites

구제역 가축 매몰지 인근 지하수의 특성과 검출 미생물 간의 연관성 연구

2013년 2월

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지도교수 고 광 표

이 논문을 보건학 석사학위 논문으로 제출함

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안경목의 석사학위논문을 인준함

2013년 2월

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ABSTRACT

Association of Microorganisms with Physico-chemical and Microbiological Characters of Ground Water near Foot-and-Mouth Disease Burial Sites

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Because of Korea FMD outbreak from Nov. 2010 to Apr. 2011, numerous burial sites of livestock carcass were made and contamination of groundwater around the burial sites was concerned. In our study, total 273 sites near the burial sites were chosen for groundwater sampling. And the groundwater was sampled once at each

site in the spring season (Mar to April) or the summer season (July to September) or the fall season (October to November) in 2011.

After inspecting indicator bacterial, pathogenic bacterial and viral contamination of the groundwater samples, total cell colony, total coliform, fecal coliform, *E. coli*, *Salmonella* spp., *Clostridium perfringens* and norovirus GⅡ were detected more than permission limit for drinking water in Korea. However, *E. coli* O157, *Shigella* spp., norovirus GⅠ and enterovirus was undetectable in any sample. The detection frequency of each microorganism was generally higher in the summer season, and we can reckon that the detection frequency of each detected microorganisms between the three seasons was significantly different.

Comparing the physico-chemical characters of groundwater seasonally, the temperature of groundwater was highest in the summer season and getting lower in the order of the fall and the spring. The temperature of groundwater in which indicator bacteria or *Clostridium perfringens* were detected seemed to be higher than that of each microorganism non-detected groundwater. When we performed chi square test to study the association between detections of each microorganism, total coliform had positive association with all the rest detected in groundwater samples. Being different from public concern, no association was observed between detection frequencies of microorganisms and the FMD livestock burial.

The present study suggests that distribution of indicator microorganisms has close relationship with temperature, and thus temperature is an important physicochemical parameter for groundwater quality management. Furthermore,

fecal coliform inspection appeared to be the effective method to predict the groundwater contamination from other indicator bacteria and pathogenic bacteria.

Keywords : groundwater, indicator microorganism, pathogen, virus, temperature, total coliform

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I. Introduction

Groundwater serves as an important source of drinking water, household and industrial water in both developing and developed countries [1]. Groundwater is generally recognized to have little risk of contamination by waterborne pathogens because various bacteria and viruses in nature are filtered and removed while surface water moves through soil in its path to the groundwater stock [2, 3]. In many regions, it has several benefits such as high availability, easiness to develop and low cost, and it demands minimal treatment prior to use [4, 5]. Thus, in 2006, groundwater covered 11% of the total water supply in South Korea [6]. However, since most groundwater is used without treatment facility, the risk of pathogens is high in case of contamination [7]. Outbreak reports that groundwater contamination caused waterborne disease outbreaks have been made in many regions globally [8, 9, 10]. For instance, in USA, nearly 40 million people per year are estimated to be infected by waterborne outbreaks caused by groundwater contamination [11]. Therefore, it is very important to correctly monitor the quality of groundwater for prevention of such outbreaks. Previous studies have documented that waterborne pathogens including microbial indicators contaminated almost 15–86% of groundwater [12, 13]. The Drinking Water Quality Standard of Korea specifies inspection items including indicator microorganisms; total colony count, total coliform, fecal coliform and *E. coli*. Moreover, *Salmonella* spp., *Shigella* spp. and *Clostridium perfringens* et cetra are also included in the items as waterborne

pathogens [14]. Epidemiological studies have reported an increased risk of acquiring gastrointestinal and respiratory diseases after contact with water having raised concentrations of coliform bacteria [15, 16]. Bacterial fecal indicators including fecal coliform and *Enterococcus* spp. are the most usually tested indicator microorganisms for water quality. [17] Viruses are especially problematical because they can easily penetrate soil because of their small size. In addition, it has been reported that human enteric viruses can be detected in groundwater with no bacterial indicators like total or thermo tolerant coliforms [10, 18, 19]. Norovirus, belonging to the Caliciviridae family, is causal agent of foodborne outbreaks of viral gastroenteritis. Among five genogroups of norovirus, genogroup GI, GII and GⅣ infect human. During epidemic seasons, the most prevalent genogroups detected in human samples are GI and GII [20, 21]. In 2006, foodborne outbreak associated with Norovirus happened in the Seoul area and infected about 2000 schoolchildren. It was the most massive foodborne outbreak recorded in the history of South Korea. An epidemiological investigation indicated contaminated groundwater was also suspected source of norovirus [22]. Enteroviruses are single-stranded RNA viruses like norovirus, but belong to the Picornaviridae family. Enteroviruses infect and make diseases in mammals inclusive of human, and are spread through the fecal-oral route. For children, they are known as the most common causes of poliomyelitis and aseptic meningitis [23].

Foot-and-mouth disease (FMD) is a viral infectious disease occurred to cloven-hoofed animals, and is characterized by blisters inside the mouth and on the feet.

In South Korea, FMD outbreak spread across the nation except Jeolla area and Jeju province from Nov. 2010 to Apr. 2011. Thousands of stockbreeding farm and nationwide economy were damaged enormously from the outbreak. Through the 153 reported cases of FMD in cows and pigs, over 3,300,000 of pigs and over 150,000 of cows were slaughtered for prevention. To dispose of the livestock's carcasses, about 4,600 burial sites were made [24]. However, because of short time to perform burial procedure, the groundwater contamination by leachate from incomplete burial sites is concerned. Increasing temperature and nitrates caused by decomposition of buried stock are beneficial to growth of *E. coli* and pathogenic microorganisms [25]. Leachate from stock burial site makes soil contamination and it may flow into groundwater after down pours. In 2001, UK Department of Health reported that *E. coli* O-157, *Salmonella, Clostridium perfringens* and other pathogens existed in the leachate flowed out from the buried carcasses [26, 27]. The report of Scottish Executive Health Department in 2001 suggested continuous environmental monitoring is required in order to manage the risk of contamination of ground water and nearby surface waters due to movement of leachate originated from the burial site of mass animal carcasses [28].

Thus, the objectives of present study were to confirm the existence of indicator microorganisms and waterborne pathogenic microorganisms in groundwater near FMD burial sites and to evaluate association between physicochemical parameters and detected microorganisms in order to give useful information for groundwater protection and efficient monitoring.

II. Materials and Methods

1. Sampling sites

Groundwater was sampled from a total of 273 sites located near burials for Foot and Mouth Disease stock in the Gyeonggi providence, the Kangwon providence, the Gyeongsang Area and the Chungcheong Area in South Korea (Fig. 1). The wells were located at various facilities including factories, military facilities, and restaurants. 191 sampling sites were selected in the Gyeonggi providence because the most number of FMD burial sites was made in this area during the last epidemic. 39 sampling sites in the Kangwon providence, 30 sampling sites in the Gyeongsang area and 13 sampling sites in the Chungcheong area were also selected. Sampling was attempted once in the spring season (March to April), the summer season (July to September) and the fall season (October to November) in 2011. 140 sites were sampled in the spring and 40 sites in the summer, 93 sites in the fall season. Thus, a total of 273 samples were taken for this study. Information of the closest burial site such as distance from sampling site, species and number of buried stocks were also collected.

FIGURE 1. Sampling sites for groundwater in Korea

2. Groundwater sampling

At some sites, sampling was performed with positive pressure pump through a hole directly connected to groundwater. On the other hand, at other sites, a water tank was installed and linked to groundwater. The stored water in the tank was sampled through a water tap. Groundwater samples were collected in two of 1-L sterile bottles for analysis of indicator microorganisms and bacterial pathogens. To detect viral pathogens in groundwater, the sampler consisted of a flow meter, a pressure gauge, a Virosorb® 1MDS filter (CUNO Inc., USA), a cartridge filter housing (Aqua-Pure, USA) and tubing, which was manufactured following the USEPA manual [17]. At most site, the water pressure of input groundwater was 30 pounds per square inch (PSI), and approximately 500 L of groundwater was sampled from those sites. However, at some sites, the water pressure was under 30 PSI and thus, more than 500L was sampled. The entire sampling device was autoclaved prior to field sampling. The valves connected to the sampler were sterilized with 70% alcohol. Actual sampling was performed after 70 L of flow. Water pH, temperature, and turbidity were determined from the first 70 L of water. After sampling, the filter housing was removed from the sampler. And along with the sampled bottles, the filter housing was immediately stored at 4 ℃ until transferred to the laboratory and was kept at 4 ℃ until analysis. Sample analysis was performed within 72 h of sampling.

FIGURE 2. Pictures of groundwater sampling using 1MDS cartridge filter for detecting viruses.
The use of these pictures is permitted by National Institute of Environmental Research, Republic of Korea.

3. Analysis of indicator microorganisms

Total colony count (TCC) was measured using the pour plate method as described in a previous study [29]. Samples were serially diluted with sterile phosphate buffer solution. Then, one mL of each of the diluted samples was poured into a Petri dish with 10–12 mL plate count agar. After incubation at 35 ℃ for 24±2 h, colony forming units (CFU) were counted. The samples were assayed for bacterial fecal indicators (total coliform, fecal coliform, and *E. coli*) using the defined substrate technology test kits, Colilert® combined Quanti-tray® 2000 (IDEXX Laboratories, USA). Incubation and enumeration from samples were conducted following the manufacturer's instructions. One hundred mL of groundwater sample was taken using a disposable bottle, and the reagent was mixed with the sample. The solution in the bottle poured into Quanti-tray and the tray was sealed. After incubation and UV radiation, the concentrations of each indicator were measured by the most probable number (MPN).

FIGURE 3. Detection of coliform bacteria using Colilert® and Quanti-tray® 2000

4. Detection of bacterial pathogens

A. *Escherichia coli* O157:H7

To detect bacterial pathogens, groundwater samples was conducted using membrane filtration according to standard method [30]. Two hundred fifty mL of groundwater samples were filtered through 0.45 μm pore-sized membrane filter (Pall Corp., USA). The filter inserted into a sterile tube containing 10 mL of mEC broth and was incubated at 35 ℃ for 24±2 h. In case total coliform or fecal coliform had been positive in the bacterial fecal indicators assay, following experiments were conducted.

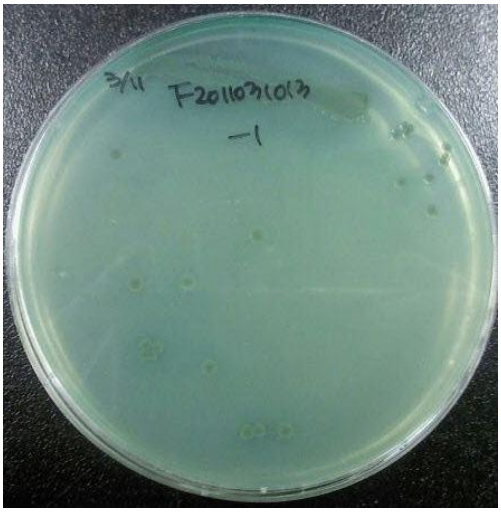
Incubated mEC broth was inoculated on MacConkey sorbitol agar medium (w/ cefixime tellurite) and incubated at 35 ℃ for 24 h. A typical *Escherichia coli* O157 colony is slightly transparent, almost colorless with a weak pale brownish appearance. The colony was streaked onto Eosin methylene blue agar plate, and the dishes were incubated at 35 ℃ for 24±2 h. Colonies showing a green color with a metallic surface sheen were inoculated on nutrient agar medium and incubated at 35 ℃ for 24 h. Individual isolates were identified biochemically as *E. coli* or *E. coli* O157 with API® 20E test strip (BioMérieux, France). Identified *E. coli* cells were taken from a nutrient agar plate and mixed with antisera (Denka Seiken, Japan) on a glass slide for confirming the presence of O157 and H7 antigens.

B. ***Salmonella* spp. and *Shigella* spp.**

After membrane filtration, the prepared filter membrane was directly placed into a sterile tube containing 10 mL of Selenite broth and was incubated at 37 ℃ for 18~24 h. This broth used for detection of both *Salmonella* spp. and *Shigella* spp. Using a sterile loop, the incubated broth was streaked on Bithmuth sulfite agar medium and incubated at 35 ℃ for 48 h. Black and sheen colonies with thin and white rim were typical form of *Salmonella* spp. Each typical colony and green colony as atypical form was streaked onto Tryptic soy agar plate, respectively. After incubation at 37 ℃ for 18~24 h, individual colonies were identified biochemically with API® 20E test strip in duplicates.

For *Shigella* spp., the incubated Selenite broth was streaked with a sterile loop on XLD agar medium and incubated at 35 ℃ for 24 h. Colonies showing a red or yellow or transparent color were moved to Tryptic soy agar plate. Further identification steps were same as ones of *Salmonella* spp.

FIGURE 4. Identification of *Salmonella* spp. by the API 20E kit



C. ***Clostridium perfringens***

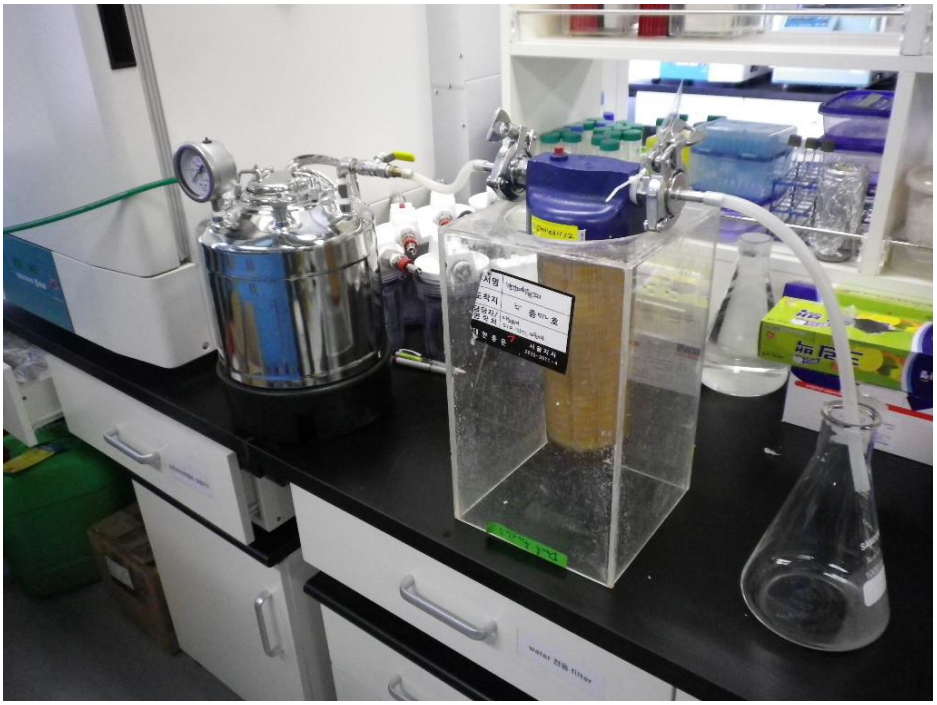
One hundred mL of groundwater samples were filtered through 0.45 μm pore-sized membrane filter and repeated to prepare two membrane filters. Each filter was directly placed onto TSC agar medium containing egg yolk emulsion. The plates were moved into anaerobic jar with anaeropack (Mitsubishi Gas Chemical company, Japan) or anaerobic chamber (Coy laboratory, USA). In anaerobic conditions, the plates were incubated at 37 ℃ for 24±2 h. Typical C. *perfringens* colonies are black, while opaque white zone surrounding creamy yellow or yellowish gray colonies are considered as atypical forms. Up to five typical colonies were selected and each was streaked on duplicate nutrient agar medium. One plate was incubated in aerobic condition at 37℃ for 24 h, and the other was simultaneously incubated in anaerobic condition at 37℃ for 24 h. Colonies grown in anaerobic condition only were moved to TSC agar medium and incubated again at 37 ℃ for 24 ± 2 h in anaerobic condition. Individual colonies were identified biochemically with API® 20A test strip (BioMérieux, France) in duplicate.

5. Detection of viral pathogens

A. Concentrations of viruses in groundwater

In order to elute viruses from the cartridge filter, the virus adsorption-elution (VIRADEL) technique used in the recovery of norovirus [31]. Briefly, the sampled filter was subjected to elution by an elution buffer composed of 1.5% beef extract (Difco, USA), 0.05M glycine, 1% tween 40 (pH 9.5). The cartridge housing was filled with elution buffer and allowed to be in contact for 30 min. Then pressurized nitrogen gas was used to force out the eluent. The eluent was subjected to acid precipitation with 1 M HCl at pH 3.5 ± 0.1 for 30 min of stirring. The precipitant was centrifuged at 2500 g at 4 ℃ for 15 min. The pellet was completely dissolved using 20 mL 0.15 M sodium phosphate (Na2HPO4•7H2O, pH 9.0–9.5). The suspension was carefully collected using a pipette. The sample was filtered through a 0.45-μm pore size syringe filter (Millipore, USA) to remove non-viral organisms and stored at −70 ℃ until analysis.

FIGURE 5. Picture of experimental setting for virus elution procedure



B. **RNA extraction and analysis of norovirus and enterovirus by RT-PCR assay**

Viral RNA was extracted from 150 μl aliquots of the viral concentrates using a QIAamp® Viral RNA mini kit (Qiagen, USA) according to the manufacturer's instructions. All RT-PCR amplification steps were performed using a Verso™ 1-Step RT-PCR Hot-Start Kit (Thermo Fisher Scientific, USA). Nested RT-PCR assays were used for each genogroup (GI and II) of norovirus (NoV) and pan-enterovirus [32, 33, 34]. Thus, three sets of RT-PCR and nested PCR were executed for each sample. The primer sets for GI NoV (GI-F1M/GI-R1M[first round], GI-F2/GI-R1M [second round]), GII NoV (GII-F1M/GII-R1M [first round], GIIF3M/GII-R1M [second round]) and enterovirus (EV1/EV2 [first round], EV1/EV3 [second round]) are described (Table 1). The RT-PCR reaction mixture (20 μL) for all viruses was composed of 2× 1-Step PCR Hot-Start Master Mix (9.5 μL), RT Enhancer (1 μL), 10 pM primers (4 uL), Verso RT enzymix (0.5 μL) and viral RNA template (5 μL). The reverse transcription reaction was incubated at 50 ℃ for 15 min. The PCR amplification program consisted of an initial 15 min denaturation step at 95 ℃, followed by 35 (NoV) or 30 (enterovirus) cycles of denaturation at 94 ℃ for 30 sec, annealing at 55 ℃ for 30 sec (NoV) or 60 ℃ for 45 sec (enterovirus), and extension at 72 ℃ for 1 min 30 sec (NoV) or 1 min (enterovirus) with a 10-min final extension at 72 ℃.

Nested PCR steps, the second rounds, were performed using EmeraldAmp® PCR Master Mix (Takara, Japan). The reaction mixture (50 μL) for all viruses contained 2 × EmeraldAmp PCR Master Mix (25 μL), 10 pM primers (4 uL), distilled water (19 μL) and RT-PCR product (2 uL). The nested PCR conditions for NoV were as follows: an initial 5-min denaturation at 94 ℃; 25 cycles of 94℃ for 30 sec , 55℃ for 30 sec and 72 ℃ for 1 min 30 sec; a 7-min final extension at 72 ℃. The thermal cycling conditions for enterovirus were as follows: 30 cycles of 98℃ for 10 sec , 60℃ for 45 sec and 72 ℃ for 1 min; 10 min at 72 ℃. To prevent cross-contamination during the nested PCR, multiple negative controls (at least one for every 10 samples) were included in addition to a positive control, which was provided by The Korea National Institute of Environmental Research. Amplified products were separated by electrophoresis on a 1.5 % agarose gel, stained with 20 μg/ml ethidium bromide (Sigma, USA) and visualized under UV light. The positive samples were further analyzed for nucleic acid sequencing and identification.

TABLE 1. Oligonucleotide primers used for detection of viruses

Viral pathogens	Primers		Sequence [a] (5' to 3')
Norovirus GI	GI-F1M	(Forward)	CTG CCC GAA TTY GTA AAT GAT GAT
	GI-R1M	(Reverse)	CCA ACC CAR CCA TTR TAC ATY TG
	GI-F2	(Forward)	ATG ATG ATG GCG TCT AAG GAC GC
Norovirus GII	GII-F1M	(Forward)	GGG AGG GCG ATC GCA ATC T
	GII-R1M	(Reverse)	CCR CCI GCA TRI CCR TTR TAC AT
	GII-F3M	(Forward)	TTG TGA ATG AAG ATG GCG TCG ART
pan-Enterovirus	EV1	(Forward)	TCC GGC CCC TGA ATG CGG CT
	EV2	(Reverse)	TGT CAC CAT AAG CAG CC
	EV3	(Forward)	CCC AAA GTA GTC GGT TCC CC

[a] A : Adenine , G: Guanine, T: Thymine, C: Cytosine, R: Adenine or Guanine, Y: Cytosine or Thymine, I: Adenine or Guanine or Cytosine or Thymine

C. **Phylogenetic analysis**

The identifications of viruses were confirmed by nucleic acid sequence analysis. After electrophoresis, the nested RT-PCR products were cut out from the gel with a razor blade and extracted using the QIAquick Gel Extraction Kit (Qiagen, USA). Sequence analysis of the products was contracted out to a commercial sequencing company (Cosmo Genetech, South Korea). The sequences were compared to those in the GenBank database using the National Center for Biotechnology Information (NCBI) BLAST search program.

6. Statistical Analysis

The occurrence of microorganisms were categorized into binary variables based on drinking water quality standards (Ministry of Environment, South Korea), as follows: a score of 1 for total colony count≥ 20 CFU/mL, total coliform ≥1 CFU/100 mL, fecal coliform ≥1 CFU/100 mL *E. coli* ≥1 CFU/100 mL, *Escherichia coli* O157:H7 ≥1 CFU/100 mL, *Salmonella* spp. ≥1 CFU/250 mL , *Shigella* spp. ≥1 CFU/250 mL, *Clostridium perfringens* ≥1 CFU/100 mL and NoV detected, pan-enterovirus detected [14]. All other samples were given a score of 0.

The prevalence of and microbial indicators, bacterial pathogens and enteric viruses in groundwater was estimated. Chi square test (Homogeneity of proportions test) was applied to confirm the difference of occurrence frequency of microorganisms among seasons. One-way ANOVA followed by the Tukey or Dunnett multiple comparisons test were used to compare physicochemical parameters among seasons. Independent samples t-test were applied to compare the levels of physicochemical parameters between microorganism positive group and negative group. Chi square test (Independence of variables test) was used to study the association between detections of each microorganism. All statistical analyses were performed employing Statistical Package for the Social Sciences (version 20.0). P values less than 0.05 were used for statistical significance.

Ⅲ. Results

1. Characteristics of sampling sites and groundwater

The characteristics of the groundwater, including sampling volume, water temperature and turbidity, are described in Table 2. Unlike samples obtained in the summer and the fall, burial site information of the samples from the spring season was not gathered. Thus, characteristics of 132 burial sites such as distance from sampling site and number of buried stocks are shown in Table 2. Species of buried stocks were divided in 3 groups (cow: 73 sites, pig: 52 sites, mixed of cow, pig, deer and goat: 7 sites).

TABLE 2. Summary of groundwater conditions and burial sites.

Statistics	Groundwater				Burial site	
	Sampled volume of water (liter)	Temperature (℃)	Turbidity (NTU)[a]	pH	Number of buried stock	Distance from sampling point (meter)
Mean	549.6	14.31	0.69	6.70	833.1	301.1
Standard Deviation	113.8	2.86	4.05	0.59	1408.8	319.1
Maximum	1426.0	25.60	53.00	8.72	7932.0	3000.0
Minimum	450.0	4.20	0.00	4.81	1.0	30.0
Median	506.0	13.90	0.00	6.67	132.0	231.0
No. of samples	273	273	273	273	132	132

[a] Nephelometric turbidity unit.

2. Incidence of microorganisms in groundwater

Contamination by total colony count (TCC), total coliform (TC), fecal coliform (FC) and *E. coli* as microbial indicators and *E. coli* O-157, *Salmonella* spp., *Shigella* spp., *Clostridium perfringens*, Norovirus G-Ⅰ, Norovirus GⅡ, and pan-Enterovirus as pathogens was analyzed. Among 273 tested samples, *E. coli* O-157, *Shigella* spp., Norovirus G-Ⅰ and pan-Enterovirus was not detected (Table 3). In the spring season, 18 (12.9%), 12 (8.6%) and 3(2.1%) were positive for TCC, TC and *E. coli*, respectively. In the summer season, TCC, TC, FC and *E. coli* were positive in 18 (45.0%), 12 (30.0%), 8 (20.0%) and 9 (22.5%) samples, respectively. In the fall season, 35 (37.6%), 45 (48.4%), 7 (7.5%) and 7 (7.5%) samples were positive, respectively. Among the 140 spring and 93 fall samples, 1 (0.7%) and 8 (8.6%) were positive for *Salmonella* spp., respectively. In the other samples among the spring and fall samples, at the same rate as *Salmonella* spp., 1 (0.7%) and 8 (8.6%) were positive for *Clostridium perfringens*, respectively. Norovirus was detected in one samples in the fall season, but not detected in the other seasons. The genogroup of norovirus detected in a groundwater sample was confirmed by nucleic acid sequencing and phylogenetic analysis. The sequence of the sample was identified in genogroup Ⅱ. Finally, the difference in microorganisms' occurrence among the three seasons was analyzed. Except norovirus, the incidence rate of each detected microorganisms between the three seasons was significantly different ($p < 0.01$).

TABLE 3. Seasonal occurrence of indicators and pathogens

indicators and pathogens	No. positive [a] (%)			p-value
	spring(Mar-Apr) (n=140)	summer(Jul-Sep) (n=40)	fall(Oct-Nov) (n=93)	(Chi -square)
Total colony count	18 (12.9)	18 (45.0)	35 (37.6)	< 0.01
Total coliform	12 (8.6)	12 (30.0)	45 (48.4)	< 0.01
Fecal coliform	0	8 (20.0)	7 (7.5)	< 0.01
E. coli	3 (2.1)	9 (22.5)	7 (7.5)	< 0.01
E. coli O-157	0	0	0	
Salmonella spp.	1 (0.7)	0	8 (8.6)	< 0.01
Shigella spp.	0	0	0	
Clostridium perfringens	1 (0.7)	0	8 (8.6)	< 0.01
Norovirus GⅠ	0	0	0	
Norovirus GⅡ	0	0	1 (1.1)	
Pan-Enterovirus	0	0	0	

[a] +, Positive: total colony count > 20CFU/mL, total coliform and fecal coliform and *E. coli* and *E. coli* O-157 and *Clostridium perfringens* ≥1 CFU/ 100 mL, *Salmonella* spp. and *Shigella* spp. ≥1 CFU/ 250 mL ;
norovirus and pan-enterovirus were detected using nested PCR and confirmed by sequence analysis.

3. Seasonal changes in physicochemical parameters

Physicochemical properties of the groundwater also varied seasonally (Table 4). The mean water temperatures of the spring, the summer and fall samples were 12.95℃ and 18.27℃ and 14.65℃, respectively. As expected, water temperature was the highest in the summer and the water temperature was significantly different between the seasons ($P < 0.01$). Particularly, following the summer season, the water temperature of the fall was higher than one of the spring. Level of pH was significantly higher ($P < 0.01$) in the early seasons(from spring to summer) than in the later seasons(from summer to fall). However, the turbidity did not significantly change through the three seasons.

TABLE 4. Seasonal difference of physicochemical parameters

	spring(Mar-Apr) (n=140)	summer(Jul-Sep) (n=40)	fall(Oct-Nov) (n=93)	P values (One way ANOVA)
Temperature (℃)	12.95 ± 1.97 c	18.27 ± 2.79 a	14.65 ± 2.29 b	< 0.01
Turbidity (NTU)	0.45 ± 3.01	0.41 ± 1.03	1.16 ± 5.83	0.384
pH	6.73 ± 0.57 a,b	6.94 ± 0.65 a	6.56 ± 0.57 b	< 0.01

Each datum represents the mean ± standard deviation. Among the three seasons for the same parameter, means with the different letters are significantly different according to one-way ANOVA. (p = 0.05)

4. Physicochemical parameters associated with incidence of microorganisms

The relationships among physicochemical parameters and microorganism occurrence were statistically analyzed (Table 5). The 273 groundwater samples were categorized into binary groups by each detected microorganism: positive group; the microorganism detected, negative group; the microorganism non-detected. The mean water temperature of the TCC positive group was 15.11℃ and that of TCC negative group was 14.03℃. The water temperature of TCC positive group was significantly higher than one of TCC negative group. Similar results were found regarding water temperature depending on the presence of TC, FC, *E. coli* and *Clostridium perfringens*. The result showed that the microorganisms were positively ($p < 0.05$) associated with temperature. To the contrary, no significant difference was found at temperature between *Salmonella* positive group and *Salmonella* negative group. There was no relation between any microorganism and other physicochemical parameters.

TABLE 5. Levels of indicators and physiochemical parameters of groundwater in microorganism positive groups

indicators and virus		Geometric mean		Temperature (℃)	Turbidity (NTU)	pH
Total colony count	(n=71)	183.48	CFU/ ml	15.11*	1.92	6.76
Total coliform	(n=69)	13.62	CFU/ 100 mL	15.49*	0.33	6.75
Fecal coliform	(n=15)	6.40	CFU/ 100 mL	17.74*	0.68	6.79
E. coli	(n=19)	4.97	CFU/ 100 mL	17.34*	0.55	6.85
Salmonella spp.	(n=9)			14.61	0.39	6.75
C. perfringens	(n=9)			17.10*	1.58	6.84
Norovirus GⅡ	(n=1)			13.40	0.00	7.62

Each datum for the physiochemical parameters represents the geometric mean.
Detections of *Salmonella* spp., *Clostridium perfringens* and norovirus conducted in qualitative methods
* attached means indicate mean of positive group is significantly higher ($p < 0.05$) than mean of negative group in light of independent t-test.

5. Association between incidences of microorganisms

Independence of variables test was performed and table 6 summarizes relation between each microorganism except norovirus. The very low P values show that two microorganisms have highly positive association. Total colony count (TCC) was positively associated with other indicator microorganisms; TC, FC and *E. coli*. In addition to the association between TCC and TC, total coliform(TC) had positive association with all the rest detected in groundwater samples. TCC, TC and *E. coli* were positively associated with fecal coliform(FC). However, TCC, FC and *E. coli* had no relation with not only *Salmonella* spp. but *Clostridium perfringens*. Despite of small number of the detected samples, *Salmonella* spp. and *Clostridium perfringens* showed positive association.

Table 6. Association matrix for detection of indicators and pathogens in groundwater samples

	Total colony count	Total coliform	Fecal coliform	*E. coli*	*Salmonella* spp.	*C. perfringens*
Total colony count	273.00					
Total coliform	**19.91 (< 0.01)**	273.00				
Fecal coliform	**30.35 (< 0.01)**	**46.93 (< 0.01)**	273.00			
E. coli	**29.74 (< 0.01)**	**60.38 (< 0.01)**	**182.87 (< 0.01)**	273.00		
Salmonella spp.	1.64 (0.200)	**13.58 (< 0.01)**	0.57 (0.452)	3.35 (0.067)	273.00	
C. perfringens	1.64 (0.200)	**8.44 (< 0.01)**	0.57 (0.452)	3.35 (0.067)	**26.34 (< 0.01)**	273.000

Each datum shows Pearson chi-square value and values in parentheses are P values of chi-square test.
Values in bold letters indicate significant association ($p < 0.05$).

Ⅳ. Discussion

Epidemic outbreak of Foot-and-mouth (FMD) disease happened in South Korea from Nov. 2010 to Apr. 2011 and millions of livestock were buried at thousands of burial sites nationwide. However, a large scale creation of burials in short time gave rise to concerns about groundwater contamination around the FMD burial sites. In this study, we investigated the biological water quality of groundwater near the burial sites following Drinking Water Quality Standard of Korea guidance. One of the main objectives of our study is to detect indicator microorganisms and pathogenic microorganisms in the groundwater. And the other is to analyze relation of a microorganism to physicochemical parameters or other detected microorganism. At last, the information obtained from this study may contribute toward groundwater management.

Among the 273 samples taken from 273 sites for this study, 31 (22.1%) ,23 (57.5%) and 56 (60.2 %) from the spring, the summer and fall seasons were positive, respectively. In all the three seasons, total cell colony(TCC), total coliform(TC), and *E. coli* were detected. Fecal coliform was observed in the summer and the fall but not in the spring. *Salmonella* spp. and *Clostridium perfringens* were found in the spring and the fall. Norovirus, identified as genogroup Ⅱ, was only detected in one groundwater samples in the fall season. In this experiment, the rest of the inspected pathogens were not detected.

Among the whole samples, incidence rates of detected microorganisms as follows:

total cell colony 26.0%, total coliform 25.3%, fecal coliform(FC) 5.5%, *E. coli* 7.0%, *Salmonella* spp. 3.3%, *Clostridium perfringens* 3.3% and norovirus 0.4%. In the summer season, most indicator bacteria shows high incidence rate in comparison with other seasons, but no pathogenic microorganism was found in this season. The result of Chi square analysis suggests that groundwater quality biologically differs by season. In contrast to indicator microorganisms, *Salmonella* spp. and *Clostridium perfringens* were further frequently detected in the fall season. After the rainy spell in summer of Korea, more microorganisms and nutrients beneficial to the growth of them might flow into groundwater. This effect of precipitation is thought to be the reason for the frequent incidence of two pathogenic bacteria during the fall season. The occurrence of norovirus in groundwater has been reported to be typically 8−21% worldwide [9, 35, 36]. Thus, the prevalence of norovirus in this study is much lower than average. However, it is important that norovirus was detected in fall season in which norovirus outbreaks occur generally frequently [22, 37].

From a one-way ANOVA to compare physicochemical parameters of groundwater between seasons, temperature and pH were significantly different by seasons. Particularly, water temperature of each season was significantly different with each other: the summer, the fall, the spring in the order of higher to lower means of temperature. Among means of turbidity, 95% confidence intervals overlapped with each other so turbidity was not changed seasonally. In case of surface water, previous study report that high nutrients due to the great runoff in the summer

season [38, 39, 40], may have stimulated the algal growth and result in high pH and turbidity in the summer season [41]. However, in this study, such effect on pH and turbidity of the groundwater did not appear.

By each microorganism, the groundwater samples divided into detected group and non-detected group so that we analyzed the difference of physicochemical parameters between two groups using independent t-test. In case of TCC, TC, FC, *E. coli* and *Clostridium perfringens*, only temperature was significantly higher in detected group. This result indicates that higher water temperature is beneficial to growth of those microorganisms and similar inference comes from Table 3: the higher groundwater temperature, the higher incidence rates of microorganisms is. Our results confirmed previous study that proper temperature is beneficial factor for the coliform survival and/or growth [42, 43]. In contrast, pH values of nearly all groundwater samples obtained in neutral region that is advantageous to growth of microorganism.

In our study, we also considered association between detected microorganisms data and burial site information such as distance from groundwater reservoir, species and number of buried stocks. However, Chi-square tests resulted that no statistical associations between them were identified (data are not shown). Thus, we could not find any relationships between the FMD livestock burial and microbial contamination of groundwater. This result supports the previous report that cannot find any effect of leachate to microbial detection in groundwater of Korea [44].

Association matrix for detection of each microorganism suggests intra-relationship between two microorganisms. TCC, FC has positive association with other indicator bacteria. It should be noted that TC is positively associated with all the detected microorganisms. Lieberman et al. [19] suggested that total coliforms have the best predictive value for the investigation of water quality. On the other hand, the present result is discordant with WHO guidance that FC is a better indicator for fecal contamination than TC [45]. This analysis determined that frequent monitoring of simple parameters, total coliforms, is the best approach to maximize the probability of detecting groundwater quality changes and the contamination of groundwater because this bacteria can be measured in simple method and can be measured at a lower cost than the detection of pathogens or other indicators and are already included in most regulations. However, since the numbers of detected pathogens are not enough to confirm association between pathogens, we cannot have confidence that some pathogens such as *Shigella* spp., norovirus G-Ⅰ and pan-enterovirus can be removed from inspect items.

In this study, we could not search seasonal change of groundwater quality at the same sampling point for sampling regions and sites are different according to the seasons. The results from a single sampling do not provide enough information to assess association between data obtained from the groundwater. Thus, further studies characterizing the microbial relation of groundwater should be required for negative controls and frequent and repeat sampling at the same points.

In conclusion, this study investigated the prevalence of important microorganisms in groundwater in aspect of human health. It can provide beneficial information to people using groundwater located near FMD burial site. From our study, Statistical analysis demonstrated that indicator bacteria distributions were closely related to a physicochemical factor, temperature. Therefore, groundwater temperature must be considered in order to manage groundwater safely. Periodic sampling of groundwater for easy-to-measure total coliform with standardized methods provides a cost effective means to detect contamination. Nevertheless, to ensure safety, investigation of pathogenic bacteria and human enteric virus is still necessary. These monitoring and further well-designed studies will promote to evaluate well contamination and to protect groundwater sources from the occurrence of disease-causing microorganisms.

Ⅴ. Reference

1. **Chapman, D.** Water quality assessments. A guide to use of biota, sediments and water in environmental monitoring. 2nd ed. London. E&FN Spon; 1996.
2. **Quanrud, D. M., S. M. Carroll, C. P. Gerba, and R. G. Arnold.** 2003. Virus removal during simulated soil-aquifer treatment. Water Res 37:753-762.
3. **Yates, M. V.** Microbial considerations in wastewater reclamation and reuse. Lancaster: Technomic Publishers; 1998. 437–88.
4. **IAH (International Association of Hydrogeologists)**. Groundwater for life and livelihoods — the framework for sustainable use [Internet]. Available from: http://www.iah.org.
5. **NGWA (National Ground Water Association)**. Groundwater use for America [Internet]. Available from: http://www.ngwa.org.
6. **K-water (Korea Water Resources Corporation)**. Water resources in Korea [Internet]. Available from: http://www.kwater.or.kr.
7. **환경부**, 가축매몰지 환경관리지침, 2010.
8. **Fong, T. T., L. S. Mansfield, D. L. Wilson, D. J. Schwab, S. L. Molloy, and J. B. Rose.** 2007. Massive microbiological groundwater

contamination associated with a waterborne outbreak in Lake Erie, South Bass Island, Ohio. Environ Health Perspect 115:856-864.

9. **Gallay, A., H. De Valk, M. Cournot, B. Ladeuil, C. Hemery, C. Castor, F. Bon, F. Megraud, P. Le Cann, and J. C. Desenclos.** 2006. A large multi-pathogen waterborne community outbreak linked to faecal contamination of a groundwater system, France, 2000. Clin Microbiol Infect 12:561-570.

10. **Locas, A., C. Barthe, B. Barbeau, A. Carriere, and P. Payment.** 2007. Virus occurrence in municipal groundwater sources in Quebec, Canada. Can J Microbiol 53:688-694.

11. **Reynolds, K. A., K. D. Mena, and C. P. Gerba.** 2008. Risk of waterborne illness via drinking water in the United States. Rev Environ Contam Toxicol 192:117-158.

12. **Chae, G. T., S. T. Yun, B. Y. Choi, S. Y. Yu, H. Y. Jo, B. Mayer, Y. J. Kim, and J. Y. Lee.** 2008. Hydrochemistry of urban groundwater, Seoul, Korea: the impact of subway tunnels on groundwater quality. J Contam Hydrol 101:42-52.

13. **USEPA (U.S. Environmental Protection Agency)**. Occurrence and monitoring document for the final ground water rule, EPA-815-R-06-012. Washington: Office of GroundWater, U.S. Environmental Protection Agency; 2000b.

14. **환경부**, 먹는물 수질기준 및 검사 등에 관한 규칙, 2012.

15. **Dewailly, E., C. Poirier, and F. M. Meyer.** 1986. Health hazards associated with windsurfing on polluted water. American journal of public health 76:690-691.

16. **Haile, R. W., J. S. Witte, M. Gold, R. Cressey, C. McGee, R. C. Millikan, A. Glasser, N. Harawa, C. Ervin, P. Harmon, J. Harper, J. Dermand, J. Alamillo, K. Barrett, M. Nides, and G. Wang.** 1999. The health effects of swimming in ocean water contaminated by storm drain runoff. Epidemiology 10:355-363.

17. **USEPA (U.S. Environmental Protection Agency)**. Improved enumeration methods for the recreational water quality indicators: Enterococci, and Escherichia coli, EPA-821-R-97-004. Washington: Office of Science and Technology, U.S. Environmental Protection Agency; 2000a.

18. **Banks, W.S.L., Klohe, C.A., and Battigelli, D.A.** 2001. Occurrence and distribution of enteric viruses in shallow ground water and factors affecting well vulnerability to microbiological contamination in Worcester and Wicomico counties, Maryland. US Geological Survey, Water Resources Investigations Report 01-4216.

19. **Lieberman, R.J., Shadix, L.C., Newport, B.S., Frebis, C.P., Moyer, M.W.N., Safferman, R.S., et al.** 2002. Microbial monitoring of vulnerable public groundwater supplies. American Water Works Association Research Foundation. Report 90914.

20. **Haramoto, E., H. Katayama, and S. Ohgaki.** 2004. Detection of noroviruses in tap water in Japan by means of a new method for concentrating enteric viruses in large volumes of freshwater. Appl Environ Microb 70:2154-2160.

21. **Jean, J., D. H. D'Souza, and L. A. Jaykus.** 2004. Multiplex nucleic acid sequence-based amplification for simultaneous detection of several enteric viruses in model ready-to-eat foods. Appl Environ Microb 70:6603-6610.

22. **KCDC (Korea Centers for Disease Control and Prevention)**. Laboratory surveillance of viral acute gastroenteritis, 2005–2008, 3(6). Public health weekly report, Korea Centers for Disease Control and Prevention; 2010. p. 90–2. Korean.

23. **CDC(Centers for Disease Control and Prevention)**. Non-Polio Enterovirus Infections [Internet]. Available from: http://www.cdc.gov/ncidod/dvrd/revb/enterovirus/non-polio_entero.htm

24. **이병오 외**, 구제역 이후 한국축산업의 새로운 진로, 한국농촌경제학회, 2011.

25. **Iserson, K.V.** In Death to dust: what happens to dead bodies? 2nd ed. Tucson, Arizona. GalenPress,Ltd. ; 2001.

26. **서울시립대학교 산학협력단**, 가축 매몰에 따른 환경오염관리방안 마련, 2008

27. **United Kingdom Environment Agency**. The Environmental Impact of the Foot and Mouth Disease Outbreak: an Interim Assessment. Environment Agency, UK. 2001.

28. **HPS(Health Protection Scotland)**. [Internet]. Available from: http://www.fmdenviroimpact.hps.scot.nhs.uk/topical/ground_enviros.htm

29. **Clesceri, L., A. Greenberg, A. Eaton**. Standards methods for the examination of water and waste water. 20th ed. Washington: American Public Health Association; 1998.

30. **APHA (American Public Health Association)**. Standard Methods for the Examination of Water and Wastewater. 20th ed. Washington, American Public Health Association;1998

31. **Lee, H., M. Kim, S. Y. Paik, C. H. Lee, W. H. Jheong, J. Kim, and G. Ko.** 2011. Evaluation of electropositive filtration for recovering norovirus in water. J Water Health 9:27-36.

32. **Hafliger, D., M. Gilgen, J. Luthy, and P. Hubner.** 1997. Seminested RT-PCR systems for small round structured viruses and detection of enteric viruses in seafood. Int J Food Microbiol 37:27-36.

33. **Kim, S. H., D. S. Cheon, J. Y. Kim, D. H. Lee, W. H. Jheong, Y. J. Heo, H. M. Chung, Y. Jee, and J. S. Lee.** 2005. Outbreaks of gastroenteritis that occurred during school excursions in Korea were associated with several waterborne strains of norovirus. J Clin Microbiol 43:4836-4839.

34. **Shieh, Y. C., K. R. Calci, and R. S. Baric.** 1999. A method to detect low levels of enteric viruses in contaminated oysters. Appl Environ Microb 65:4709-4714.

35. **Borchardt, M. A., P. D. Bertz, S. K. Spencer, and D. A.** Battigelli. 2003. Incidence of enteric viruses in groundwater from household wells in Wisconsin. Appl Environ Microb 69:1172-1180.

36. **Gabrieli, R., F. Maccari, A. Ruta, A. Pana, and M. Divizia.** 2009. Norovirus Detection in Groundwater. Food Environ Virol 1:92-96.

37. **Lindesmith, L., C. Moe, S. Marionneau, N. Ruvoen, X. Jiang, L. Lindblad, P. Stewart, J. LePendu, and R. Baric.** 2003. Human susceptibility and resistance to Norwalk virus infection. Nat Med 9:548-553.

38. **Tong, S. T., and W. Chen.** 2002. Modeling the relationship between land use and surface water quality. J Environ Manage 66:377-393.

39. **Faithful, J., and W. Finlayson.** 2005. Water quality assessment for sustainable agriculture in the Wet Tropics--a community-assisted approach. Mar Pollut Bull 51:99-112.

40. **Park, M. H., X. Swamikannu, and M. K. Stenstrom.** 2009. Accuracy and precision of the volume-concentration method for urban stormwater modeling. Water Res 43:2773-2786.

41. **van der Steen, P., A. Brenner, Y. Shabtai, and G. Oron.** 2000. Improved fecal coliform decay in integrated duckweed and algal ponds. Water Sci Technol 42:363-370.

42. **LeChevallie, M.W.** Conditions favouring coliform and HPC bacterial growth in drinkingwater and on water contact surfaces. In: Heterotrophic Plate Counts and Dinking-water Safety. London, IWA Publishing; 2003. 177–197.

43. **Medema G. J., S. Shaw, M. Waite, M. Snozzi, A. Morreau, W. Grabow.** Catchment characterization and source water quality. In: Assessing Microbial Safety of Drinking Water: Improving Approaches and Methods. London, WHO OECD; 2003. 111–158.

44. **국립환경과학원**, 가축 매몰지 주변 지하수 병원성미생물 조사, 2011.

45. **WHO (World Health Organization)**. Guidelines for Drinking-water Quality. 3rd ed. Switzerland, World Health Organization Press;2006

VI. 국문초록

구제역 가축 매몰지 인근 지하수의 특성과 검출 미생물 간의 연관성 연구

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안 경 목

지도교수 고 광 표

2010년 말부터 2011년 4월 사이에 한국에서는 수많은 구제역 관련 가축 매몰지가 단기간에 조성되어, 매몰지 주변 지하수의 수질에 대한 국민들의 우려가 존재한다. 이에 본 연구에서는 매몰지 인근의 지하수 관정 273개를 선정하여, 2011년 봄, 여름, 가을 중에 관정 별로 한 번씩 지하수를 채수하였다. 지표미생물과 병원성 미생물 검사를 통해, 지하수의 미생물학적 오염도를 확인한 결과, 중온 일반세균, 총대장균군, 분원성 대장균군과 대장균, 살모넬라, 클로스트리디움 퍼프린지엔스, 노로바이러스 GⅡ가 먹는 물 수질기준을 넘어서는 수준으로 검출되었다. 각 미생물의 검출빈도는 대체로 여름에 그 빈도가 높았고, 계절에 따라 각 미생물의 검출 빈도가 통계적으로 유의하게 차이가 있음을 확인했다. 반면에, 대장균 O-157, 쉬겔라, 노로바이러스 ＧⅠ, 엔테로바이러스는 검출되지 않았다. 지하수의 물리적 특성을 계절별로 비교해보았을 때, 지하수의 온도는 여름, 가을, 봄 순서로 높게 나타났다. 지표 미생물, 클로스트리디움 퍼프린지엔스가 검출되는 지하수는 각 미생물이 검출되지 않는 지하수에 비해 온도가 높았다. 검출된 미생물들 사이의 연관성을 파악해 보았을 때, 총대장균군은 다른 모든 검출 미생물과 양의 연관 관계를 나타내었다. 미생물의 검출 빈도는 관정 인근의 매몰지의 특성과는 연관성이 발견되지 않았다. 본 연구에 따르면, 지표 미생물의 분포는 온도와 밀접한 관계를 가지고 있어, 앞으로의 지하수 관리에 있어서 중요한 물리적 특성이다. 또한, 총대장균군 시험법은 지하수의 다른 지표미생물과 병원성 미생물의

오염여부를 예상할 수 있는 효율적인 방법임을 제시하고 있다.

주요어 : 지표미생물, 지하수, 병원성 미생물, 바이러스, 온도, 총대장균군

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