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Host-specific *Bacteroides* species-based Microbial Source Tracking in South Korea

숙주 특이적 *Bacteroides* 종 기반 국내 미생물 오염원 추적 연구

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Abstract

Host-specific *Bacteroides* species-based Microbial Source Tracking in South Korea

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Various waterborne pathogens are mainly originated in human or animal feces and can cause severe and wide gastroenteric outbreaks. *Bacteroides* spp. which exhibit strong host- or group- specificities are the promising markers to identify fecal sources and their origins. In this study, a total of 240 water samples were collected from two major aquaculture areas, Aphae Island and the Goseong Bay, in South Korea, and the mean concentration and occurrence of four host-specific *Bacteroides* markers, including human, poultry, pig and ruminant, were evaluated in water samples. Moreover, we predicted potential fecal sources using *Bacteroides* markers-combined geospatial analysis, and revealed the relationship between noroviruses and human-specific Bacteroides markers. The results showed that host-specific Bacteroides markers were widely detected in study areas and poultry-specific Bacteroides marker was detected with the highest concentration (1.2 \log_{10} copies/L in Aphae Island and 1.0 \log_{10} copies/L in the Goseong Bay, respectively). In addition, from September to December 2015, the concentration of host-specific Bacteroides markers was relatively high, supposed that the low water temperature could affect the persistence of Bacteroides 16S rRNA genes. Host-specific Bacteroides markerscombined geospatial map also revealed the up-to-downstream transition of fecal contamination and the effect of land-use pattern on the concentration of host-specific Bacteroides markers. Compared to traditional bacterial indicators, human-specific Bacteroides marker showed a significant correlation with human noroviruses (r=0.337; P<0.001). Therefore, host-specific *Bacteroides* genetic markers with advanced geospatial analysis could be useful to track fecal sources in water environments.

Key words: Fecal contamination, Microbial source tracking, Host-specific *Bacteroides* markers, Noroviruses, Geographic Information System

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CONTENTS

AB	S7	ГRАСТ I
LIS	ST	OF TABLESV
LIS	ST	OF FIGURESVI
I.		Introduction 1
11.		Materials and Methods 5
	1.	Sampling sites and Water samples 5
	2.	Water collection and Sample pretreatment
	3.	Quantification of <i>Bacteoides</i> markers9
		3.1. DNA extraction and PCR assays
		3.2. Cloning and quantification
		3.3. Real-time quantitative PCR assays
	4.	Analysis of noroviruses by real-time RT-PCR assay14
	5.	Detection of total and fecal coliforms15
	6.	Host-specfic Bacteroides markers-combined geospatial
		analysis15
	7.	Statistical analysis16

III.	Results17
1.	The concentration and occurrence of host-specific <i>Bacteroides</i> markers in water samples
2.	Seasonal and spatial variations in host-specific <i>Bacteroides</i> markers
3.	Host-specific <i>Bacteroides</i> markers-combined geospatial analysis
4.	Concentration of noroviruses and coliforms in surface water samples
5.	Correlation among noroviruses, host-specific <i>Bacteroides</i> markers, and coliforms in surface water samples
IV.	Discussion
V.	References
VI.	국문초록44

List of Tables

 Table 1. Primers and probes used for the real-time PCR in this

 study
 12

List of Figures

Figure 1. Sampling sites in this study7

Figure 4. Host-specfic *Bacteroides* markers-combined geospatial map including land-use patterns in Aphae Island.......27

I. Introduction

Feces are the important water contaminants because they contain various microbial pathogens. Waterborne pathogens originated feces, including human noroviruses and food-poisoning bacteria, can cause severe gastroenteric diseases, and spread widely (1–3). Waterborne disease cases have been increasingly reported in South Korea since 2009, and 422 cases of waterborne disease outbreaks, which accounted for 8% of total infectious disease cases, occurred in South Korea in 2015 (4, 5). The South Sea is the main aquaculture area in South Korea, produced over 70% of domestic seafood (6). Various fecal sources, including sewage, farm, wildlife habitat, and harbors, can occur fecal contaminations of fisheries and shellfish farms, mostly located near the coasts of South Sea (7–9).

Microbial source tracking (MST) is worldwide-applied environmental monitoring method to monitor the fecal sources in aquatic environments using a variety of indicator microorganisms (10– 12). For precise MST studies, the selection of microbial indicators, which are highly correlated with fecal sources, are important (13). Fecal indicator bacteria, such as total coliforms, fecal coliforms and enterococci, are commonly used for water quality management worldwide (14). Human are widely known to include human-specific pathogens, but animals are also important carriers or reservoirs of enteric human pathogens, such as *Salmonella* spp., enterohemorrhagic *Eschericia coli* or *Vibrio* spp. (15, 16). Coliforms and enterococci may not be suitable for informing the origins of fecal sources due to their existences in both human and animal feces (15-18). Therefore, the host-specific microbial indicators are necessary to track fecal sources more accurately (19, 20).

Bacteroides spp. are obligate anaerobic bacteria and the large amount of *Bacteroides* spp. were detected in the feces of warm-blooded animals (21). Previous studies have shown that several *Bacteroides* spp. have the strong host- or group- specificities, and 16S rRNA genes of host-specific *Bacteroides* spp. are the promising markers to identify the origins of fecal sources, including human and animals (22, 23). Moreover, *Bacteroides* are suitable for presuming the lapse of the fecal inflows in water environments due to their short duration times after releasing from hosts (24). Additionally, human-related indicators have been suggested as alternatives to indicate the presence of human enteric viruses (25). In particular, human noroviruses are recognized as the leading human enteric viruses, and a major cause of non-bacterial acute gastroenteritis in Korea (3). Although molecular assays have been developed to directly detect enteric viruses, there are difficulties due to the diversity of viral pathogens, the need for large volumes, and the low concentrations pathogens in water samples (26). Human-specific *Bacteroides* marker could be the indicator for enteric viruses, because they have relatively great sensitivity and specificity to human fecal contamination (25, 27).

Fecal contaminants in water environments are affected by surrounding areas (28). Recently, MST can predict potential fecal contaminants more precisely and systemically via the geographic information system (GIS) with big data, including populations, land covers and land use patterns (29, 30). Especially, the land use pattern provide first-hand information on the spatial variation of the study area and fecal sources within land covers (30). Therefore, the objectives of this study were (i) to identify the distribution of host-specific *Bacteroides* markers in surface water and seawater samples from two areas of South Sea in South Korea; (ii) to predict the potential fecal sources using *Bacteroides* markers-combined geospatial map with GIS system; and (iii) to investigate the relationship between human noroviruses, containing genotype GI and GII, and microbial indicators including human-specific *Bacteroides* marker and coliforms.

II. Materials and Methods

1. Sampling sites and water samples

Aphae Island (a) and the Goseong Bay (b), located on the southern coast of South Korea, exhibit distinct geographic characteristics (Fig. 1). Aphae Island, located in Jeollanam-do, has a very rugged coast and approximately 7,000 of total residents (Fig. 1[a]). Several intensive livestock farms, breeding approximately 51,400 of poultry, 8,900 of pigs, and 2,900 of ruminants are operated in the watershed of Aphae Island (31). The daily capacity of sewage treatment facilities and public sewage treatment connection rates, presented as the percent of the population, are 70 m^3 /day and 19.6%, respectively (32). The Goseong Bay, located in Gyeongsangnam-do, exhibit an enclosed geographical feature, and the seawater inflows relatively slow because of narrow entrance (Fig. 1[b]). The Goseong Bay is the major area of ovster cultivation, occupied around 13% of oyster production in South Korea, and has approximately 26,000 of total residents (6, 33). Intensive livestock farms, breeding approximately 105,000 of poultry, 8,100 of pigs, and 2,500 of ruminant, are mostly located in the watershed of the Goseong Bay (34). The daily capacity of sewage treatment facilities

and public sewage treatment connection rates are $13,000 \text{ m}^3/\text{day}$ and 85.5%, respectively (32).

In this study, a total of 240 water samples were collected 6 times from March 2015 to January 2016 from various surface and seawater sampling sites located in Aphae Island and the Goseong Bay (Fig 1). Twenty sampling sites (10 surface water and 10 seawater sampling sites) were selected in Aphae Island (Fig. 1[a]) and 11 surface water and 9 seawater sampling sites were selected in The Goseong Bay with the consideration of the stream sites and sampling locations (Fig 1[b]).



Fig. 1. Sampling sites in this study: (a) Aphae Island; (b) the Goseong Bay. The names of sampling site were comprised of a combination of sampling site, water type, stream site and number indicating sampling location. The uppercase U and S indicate surface water and seawater, respectively.

2. Water collection and Sample pretreatment

Water samples were collected in sterilized bottles, and filtered using polyvinylidene difluoride membranes (0.22- μ m pore size, 47-mm diameter; Millipore, Cork, Ireland). The filters were kept at -80°C until nucleic acid extraction using PowerWater[®] DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The final eluents (100 μ L each) were stored at -20°C until use.

To detect human noroviruses, 100L of each water sample was filtered using NanoCeram[®] cartridge filters (Argonide Corporation, Sanford, FL, USA) as previously described with minor modifications (35). To elute noroviruses from the filters, 0.5 L of elution buffer, containing 1.5% beef extract (BD Bioscience, San Jose, CA, USA), 0.05 M glycine (Duchefa, St. Louis, MO, USA) and 1 M NaOH (pH 9.5), was used. After 5 min of incubation, the pH of eluent adjusted to 3.5 using 1 M HCl and the precipitate was acquired via centrifugation at 2,500 ×*g* for 15 min at 4°C. The precipitate was completely resuspended using 0.15 M sodium phosphate (pH 9.0–9.5), and centrifugated at 10,000 ×*g* for 10 min at 4°C. After adjusting to pH 7.0-7.5 using 1 M HCl, the supernatant was filtered using 0.22- μ m pore size syringe filter (Millipore, Bedford, MA, USA) and stored at -80°C.

3. Quantification of *Bacteroides* markers

3.1 DNA extraction and PCR assays

The human-specific, *Bacteroides dorei* (KCTC 5446; Korean Collection for Type Cultures, Jeongeup, Korea) was cultivated in tryptic soy agar (BD Biosciences, Franklin Lakes, NJ, USA) with hemin (Sigma Chemical Co. St Louis, MO, USA) and menadione (Sigma Chemical Co. St Louis, MO, USA) under anaerobic condition at 37°C. The bacteria were harvested via centrifugation at 14,000 ×*g* for 5 min at 4°C. Bacterial genome was extracted using the G-spinTM Genomic DNA extraction kit for Bacteria (Intron Biotechnology, Seongnam, Korea) according to the manufacturer's instructions and stored at -20°C until use.

The genomes of animal-specific *Bacteroides* were extracted via 200 mg of chicken, pig and cow fecal samples. Bacterial genomes from each animal fecal sample were extracted with the QIAamp[®] DNA Stool Mini Kit (QIAGEN, Hilden, Germany) with minor modifications: Briefly, the inhibitor EX buffer-treated (1 mL) fecal samples were heated for 5 min at 95°C, and bead-beated with 0.1 mm beads for 10 min, as previously described (36). The bacterial genomes were subjected to PCR using ABI PRISM GeneAmp PCR system 9700

(Applied Biosystems, Forster City, CA, USA). Table 1 summarizes the primers and probes for specific *Bacteroides* markers in this study. The reaction mixture consisted of 2.5 μ L template DNA, 2.5 μ L of 10× PCR buffer with 25 mM MgCl₂, 1 μ L of 10 mM dNTP mix, 0.25 μ L of Taq polymerase (Cosmo Genetech corporation, Seoul, Korea). PCR was performed under the following conditions: initial denaturation step at 95°C for 3 min, 30 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, extension at 72°C for 45 s, and final extension at 72°C for 3 min.

3.2 Cloning and quantification

PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and ligated into the pGEM-T Easy Vector System (Promega Corporation, Madison, WI, USA) with *E.coli* DH5 α cells according to the manufacturer's instructions. The plasmid DNA, contained host-specific *Bacteroides* markers, was extracted to the transformed cells using a Labopass Plasmid DNA Purification Kit (Cosmo Genetech Corporation, Seoul, Korea). The plasmid DNA concentrations were measured using a Nanodrop spectrophotometer ND 1000 (NanoDrop Technologies, Wilmington, DE, USA), and the total copies of marker were computed for quantifying the *Bacteroides* markers, as previously described (37).

Target	Primer and probe	e Sequence (5^{-3})	Final conc (nmol)	Reference
Human-specific Bacteroides	HF183_F HF183_R	ATCATGAGTTCACATGTCCG TACCCCGCCTACTATCTAATG	$1000 \\ 1000$	(38)
I I.	HF183_P	(FAM) CTGAGAGGAAGGTCCCCCCCACATTGGA (TAMRA)	80	
Poultry-specific Bacteroides	$qCD362_F$ $qCD464_R$	AATATTGGTCAATGGGCGAGAG CACGTAGTGTCCGTTATTCCCTTA	200 200	(17)
1	qCD394_P	(FAM) TCCTTCACGCTACTTGG (NFQ-MGB)	200	
Pig-specific Bacteroides	Pig-2-Bac_F Pig-2-Bac_R	GCATGAATTTAGCTTGCTAAATTTGAT ACCTCATACGGTATTAATCCGC	300 300	(15)
4 9	Pig-2-Bac_P	(VIC) TCCACGGGATAGCC (NFQ-MGB)	200	
Diminant manific Rantawidae	BacR_F BacR_P	GCGTATCCAACCTTCCCG	100	(10)
	Back_N Back_P	(FAM) CTTCCGAAGGGGGGGATT (NFQ-MGB)	100	(17)
-	COGIF	CGYTGGATGCGNTTYCATGA	400	
Norovirus GI	CUGIK RING1(a)-TP	CITAGACGCCATCATCATTTAC (FAM) AGATYGCGATCYCCTGTCCA (TAMRA)	400 200	(65)
	BPO-13	AICCIATGTTYAGITGGATGAG	400	
November 611	BPO-13N	AGTCAATGTTTAGGTGGATGAG	400	
TID SHITADION	BPO-14	TCGACGCCATCTTCATTCACA	400	(40)
	BPO-18	(VIC) CACRTGGGAGGGGGGGATCGCAATC (TAMRA)	200	

Table 1. Primers and probes used for the real-time PCR in this study

12

3.3 Real-time quantitative PCR assays

To determine the concentration of *Bacteroides markers*, real-time quantitative PCR was performed using the Applied Biosystems 7300 Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The reaction mixture was consisted with 2 μ L template DNA, 10 μ L of 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), and appropriate final concentration of each primer and probe (Table 1). Real-time PCR was performed in duplicate under the following conditions: 1 cycle at 95°C for 10 min; 45 cycles of 95°C for 15 s and 60°C for 1 min. All water samples were diluted 10-fold to remove PCR inhibitors. For internal quality control, negative controls (no template DNA) were performed for each experiment.

4. Analysis of noroviruses by real-time RT-PCR assay

To estimate the concentration of norovirus genogroup I (GI) and genogroup II (GII) in surface and seawater samples, duplex real-time reverse transcriptase PCR was performed (41). Viral nucleic acid extraction was performed by using the QIAamp[®] Viral RNA Mini Kit Hilden, Germany) according to the manufacturer's (Qiagen, instructions. The RT-qPCR assays were executed with a C1000 Thermal Cycler CFX96 Real-time PCR System (Bio-Rad, Hercules, CA, USA). The RT-qPCR reaction mixture was consisted with 5 µL template RNA, 400 nM of each primer (Table 1), 200 nM of each probe (Table 1), 12.5 μ L of 2 × RT-PCR buffer, 0.5 μ L of 25 × RT-PCR enzyme mix, 1.5 µL of detection enhancer using the Agpath-ID One-Step RT-PCR Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The nucleic acid was incubated at 45°C for 30 min for reverse transcription reaction. RT-qPCR was performed under the following conditions: initial denaturation step at 95°C for 10 min, 45 cycles of 95°C for 10 s and 56°C for 1 min. Ten-fold diluted norovirus RNA positive controls (AccuPower[®] Norovirus Real-time RT-PCR Kit; Bioneer, Daejeon, Korea) were used to quantify the viral copy number.

5. Detection of total and fecal coliforms

Total and fecal coliforms in surface and seawater samples were determined by using standard protocol of Most Probable Number (MPN) method (42). Five-tube of lauryl tryptose broth (Becton, Dickinson and Company, Sparks, MD, USA) were inoculated with diluted water samples and incubated at 35°C for 48 h. To distinguish between total and fecal coliform, gas-produced samples were re-inoculated into brilliant green bile broth (Oxoid, Hampshire, UK) at 35°C for 48h and into *E.coli* (EC) broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 44.5°C for 24 h. The coliform levels were calculated based on MPN table (43).

6. Host-specfic *Bacteroides* markers-combined geospatial analysis

Host-specfic *Bacteroides* markers-combined geospatial analysis was performed using ArcGIS 10.2.2 (ESRI, Inc., Redlands, CA, USA). The thematic maps containing administrative divisions, land-use pattern, and watersheds near the sampling sites were gained from the Korea Water Resources Management Information System (<u>http://www.wamis.go.kr</u>).

7. Statistical analysis

Prior to the statistical analyses, the concentration of *Bacteroides* marker, noroviruses and coliforms was log-transformed (log_{10} (copies+1)), as described previously (44). The data were expressed as means±standard deviation, and properly analyzed using Mann-Whitney *U* test. P values <0.05 indicated statistical significance. For correlation analyses between noroviruses and microbial indicators, Spearman's correlation coefficient (*r*) was calculated. IBM[®]SPSS[®] ver. 18.0.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) were used for data analyses.

III. Results

1. The concentration and occurrence of host-specific *Bacteroides* markers in water samples

Table 2 summarizes the concentration and occurrence of hostspecific *Bacteroides* markers in water samples from two sampling areas. Overall, in both areas, surface water samples showed higher mean concentration of host-specific Bacteroides markers compared to seawater samples. Both surface and seawater samples from Aphae Island showed statistically higher mean concentration of humanspecific *Bacteroides* marker than those from the Goseong Bay samples $(P \le 0.05)$. The surface water samples from Aphae Island showed the highest mean concentration (1.1 \log_{10} copies/L) and occurrence (25%) of human-specific Bacteroides marker, respectively. Moreover, the poultry-specific *Bacteroides* marker was detected over 35% in all types of water samples. Especially, the surface water samples from Aphae Island showed the highest mean concentration $(1.2 \log_{10} \text{ copies/L})$ and occurrence (50%) of poultry-specific Bacteroides marker, respectively. The mean concentration of pig-specific Bacteroides marker was similar in all water types collected from each area. The ruminant-specific Bacteroides marker was rarely detected in all water samples.

		Aphae	Island			The Gosed	ong Bay	
Host-specific	Surface	water (n=60)	Seawat	ter (n=60)	Surface v	vater (n=66)	Seawa	ter (n=54)
Bacteroides markers	Mean ^a ±SD ^b (range)	Occurrence (%)	Mean±SD (range)	Occurrence (%)	Mean±SD (range)	Occurrence (%)	Mean±SD (range)	Occurrence (%)
Human-specific	1.1±1.9 (ND ^c -5.7)	15 (25)	0.8±1.5 (ND-4.0)	14 (23)	0.6±1.3 (ND-4.2)	10 (15)	0.3±1.0 (ND-3.7)	6 (11)
Poultry-specific	1.2±1.3 (ND-3.2)	30 (50)	0.9±1.2 (ND-3.4)	21 (35)	1.0±1.4 (ND-4.2)	25 (38)	0.9±1.2 (ND-3.1)	21 (39)
Pig-specific	0.7±1.3 (ND-4.6)	13 (22)	0.5±1.1 (ND-3.1)	10 (17)	0.6±1.3 (ND-3.7)	12 (18)	0.5±1.1 (ND-3.2)	10 (19)
Ruminant-specific	0.1±0.6 (ND-3.2)	2 (3)	ND°	0 (0)	0.2±0.8 (ND-3.5)	4 (6)	0.1±0.5 (ND-2.9)	2 (4)
^a Unit : log ₁₀ copy numt	jer/L							

^b SD: Standard deviation

 $^{\circ}$ ND: not detected

18

2. Seasonal and spatial variations in host-specific *Bacteroides* markers

Figure 2 shows the mean concentration and occurrence of hostspecific *Bacteroides* markers of surface water samples in each sampling time. The surface water samples from Aphae Island showed higher concentration of human-specific Bacteroides marker than the samples from the Goseong Bay in March 2015 and from September 2015 to January 2016 (Fig. 2[a]). Especially, the surface water samples from Aphae Island showed the highest mean concentration $(2.4 \log_{10})$ copies/L) and occurrence (60%) (6 of 10) of human-specific Bacteroides marker in December 2015, respectively, significantly higher than the Goseong Bay samples (P < 0.05). The surface water samples from Aphae Island showed the highest occurrence (90%) (9 of 10) of poultry-specific Bacteroides marker in September 2015 to December 2015 and showed higher mean concentration of poultryspecific Bacteroides marker than the Goseong Bay samples over all sampling times with an exception of the samples in July 2015 (Fig. 2[b]). The surface water samples from the Goseong Bay showed 1.8 log₁₀ copies/L of poultry-specific *Bacteroides* marker in July 2015, which were significantly higher than Aphae Island samples (P < 0.01).

Over 1.0 log₁₀ copies/L of pig-specific *Bacteroides* marker was detected in surface water samples of both sampling areas in September to December 2015 (Fig. 2[c]). The ruminant-specific *Bacteroides* marker was rarely detected in the surface water samples during all of sampling periods (Fig. 2d).

Figure 3 shows the mean concentration and occurrence of hostspecific Bacteroides markers in seawater samples over all sampling times. The seawater samples from Aphae Island showed higher concentration of human-specific Bacteroides marker than those from the Goseong Bay overall sampling times with the exception of samples in December 2015 (Fig. 3[a]). In September 2015, the seawater samples from Aphae Island showed the highest mean concentration $(1.6 \log_{10})$ copies/L) and occurrence (60%) (6 of 10) of human-specific *Bacteroides* marker. Besides, the seawater samples from the Goseong Bay showed higher concentration of poultry-specific Bacteroides marker than Aphae Island samples in May to December 2015 (Fig 3[b]). Especially, in September 2015, the seawater samples from the Goseong Bay showed the highest mean concentration (2.5 \log_{10} copies/L) and occurrence (100%) (9 of 9) of poultry-specific Bacteroides marker. The seawater samples from the Goseong Bay showed the highest mean

concentration (1.9 \log_{10} copies/L) and occurrence (67%) (7 of 9) of pigspecific *Bacteroides* marker, which were significantly higher than the Aphae Island (*P*<0.05) (Fig. 3[c]). Only a few seawater samples were ruminant-specific *Bacteroides* marker-positive (Fig 3[d]).



Fig. 2. The mean concentration (**m**, Aphae Island; **m**, the Goseong Bay) and occurrence of host-specific *Bacteroides* markers (**O**, Aphae Island; **O**, the Goseong Bay) in surface water samples over sampling periods: (a) human-specific Bacteroides marker; (b) poultry-specific Bacteroides marker; (c) pigspecific *Bacteroides* marker; (d) ruminant-specific *Bacteroides* marker. Data are expressed as the mean \pm standard deviation (SD), and asterisks indicate statistical significance (*: P<0.05; **: P<0.01; Mann-Whitney U test).



Fig. 3. The mean concentrations (\blacksquare , Aphae Island; \blacksquare , the Goseong Bay) and occurrence of host-specific *Bacteroides* markers (\bigcirc , Aphae Island; \bigcirc , the Goseong Bay) in seawater samples over sampling periods: (a) human-specific Bacteroides marker; (b) poultry-specific Bacteroides marker; (c) pig-specific Bacteroides marker; (d) ruminant-specific Bacteroides marker. Data are expressed as the mean \pm SD, and asterisks indicate statistical significance (*: P<0.05; Mann-Whitney U test).

3. Host-specific *Bacteroides* markers-combined geospatial analysis

Figure 4 summarizes the results of the mean concentration of hostspecific Bacteroides markers-combined geospatial analysis containing land-use patterns in Aphae Island. Especially, the surface sampling sites in Stream B, located near the residential area, showed the higher mean concentration of human-specific *Bacteroides* marker (Fig 4[a]); Site AUb1 and AUb2 showed 2.7 log₁₀ copies/L and 2.8 log₁₀ copies/L of human-specific Bacteroides marker, respectively. Site AS7 and AS10 showed over 2.0 log₁₀ copies/L of human-specific *Bacteroides* marker and the highest occurrence (67%) (4 of 6). The surface water sampling sites, located in Stream A and B, showed over $1.0 \log_{10}$ copies/L of poultry-specific Bacteroides marker with an exception of Site AUa3 (Fig. 4[b]). Site AS3, located near Stream B showed the highest poultry-specific *Bacteroides* marker concentration (2.1 log₁₀ copies/L) with 83% (5 of 6) of occurrence. Additionally, Site AS7 and Site AS10 showed over 1.0 log₁₀ copies/L of poultry-specific *Bacteroides* marker. Site AUb1 and Site AUb2, located in Stream B, showed more than 1.0 log₁₀ copies/L of pig-specific *Bacteroides* marker (Fig. 4[c]). Especially, Site AUc2, located near the pig barn, showed 1.9 log₁₀ copies/L of pigspecific *Bacteroides* marker with 50% (3 of 6) of occurrence and Site AS10, located near Stream C, showed the highest pig-specific *Bacteroides* marker concentration (2.0 \log_{10} copies/L) with 67% (4 of 6) of occurrence.

Figure 5 presents the results of the mean concentration of hostspecific Bacteroides markers-combined geospatial analyses in the Goseong Bay. Site GUb3, located at the downstream of the residential area, showed the highest mean concentration of human-specific Bacteroides marker (1.7 \log_{10} copies/L) with 50% (3 of 6) of occurrence (Fig. 5[a]). The water surface sampling sites, located in Stream A and B, showed over 1.0 log₁₀ copies/L of poultry-specific Bacteroides marker with an exception of Site GUb3 (Fig. 5[b]). Especially, Site GUa2 showed the highest mean concentration of poultry-specific *Bacteroides* marker (2.1 \log_{10} copies/L) with 67% (4 of 6) of occurrence. Moreover, Site GS1 and Site GS5, located near Stream A and B, showed over 1.0 \log_{10} copies/L of poultry-specific Bacteroides marker and 50% (3 of 6) of occurrence, respectively. Three seawater sampling sites (GS7, GS8, and GS9), located near stream C and stream D, showed over 1.0 \log_{10} copies/L of poultry-specific *Bacteroides* marker. Additionally, Site GUa3 showed the highest mean

concentration of pig-specific *Bacteroides* marker (1.6 \log_{10} copies/L) with 50% (3 of 6) of occurrence (Fig. 5[c]). The water sampling sites, located in stream C, showed over 1.0 \log_{10} copies/L of pig-specific *Bacteroides* marker. Site GUa2, located in Stream A, showed the highest concentration ruminant-specific *Bacteroides* marker (1.1 \log_{10} copies/L) with 33% (2 of 6) of occurrence (Fig. 5[d]).



Fig. 4. Host- specific Bacteroides markers-combined geospatial map including land-use patterns in Aphae Island: (a) human-specific Bacteroides marker; (b) poultry-specific Bacteroides marker; (c) pig-specific Bacteroides marker; (d) ruminant-specific Bacteroides marker.



Fig. 5. Host-specific Bacteroides markers-combined geospatial map including land-use patterns in the Goseong Bay: (a) human-specific Bacteroides marker; (b) poultry-specific Bacteroides marker; (c) pig-specific Bacteroides marker; (d) ruminant-specific Bacteroides marker.

4. Concentration of noroviruses and coliforms in surface water samples

Table 3 summarizes the concentration and occurrence of noroviruses and coliforms in surface water samples. The surface water samples from Aphae Island showed statistically higher mean concentration of noroviruses compared to the Goseong Bay samples (P<0.001), which were 1.4 log₁₀ copies/L of norovirus GI with 37% (22 of 60) of occurrence and 1.9 log₁₀ copies/L of norovirus GII with 43% (26 of 60) of occurrence. In contrast, the mean concentration of coliforms was higher in the Goseong Bay than in Aphae Island (P>0.05). Noroviruses and coliforms were not detected in all seawater samples.

	Aphae i	sland	The Gose	ong bay
Types	Surface wat	er (n=60)	Surface wa	ter (n=66)
M	Mean±SD (range)	Occurrence (%)	Mean±SD (range)	Occurrence (%)
Norovirus GI ^a 1	1.4±1.9 (ND-5.6)	22 (37)	0.8±1.7 (ND-5.9)	13 (20)
Norovirus GII ^a	1.9±2.2 (ND-5.5)	26 (43)	0.8±1.6 (ND-5.7)	13 (20)
Total coliforms ^b	4.3±1.2 (1.7-6.2)	60 (100)	4.4±1.1 (1.3-8.2)	66 (100)
Fecal coliforms ^b	3.2±1.4 (1.3-6.2)	60 (100)	3.8±1.3 (1.3-8.2)	66 (100)

Table 3. The concentration and occurrence of noroviruses and coliforms in surface water samples of two sampling areas

30

5. Correlation among noroviruses, host-specific *Bacteroides* markers, and coliforms in surface water samples

Table 4 shows the results of the correlation analyses between noroviruses and microbial indicators containing host-specific *Bacteroides* markers and coliforms. The concentration of noroviruses (GI and GII) were significantly correlated with the concentration of human-specific *Bacteroides* marker (r=0.337; P<0.001) compared to total (r=0.304; P<0.01) or fecal coliforms (r=0.223; P<0.01). On the other hand, noroviruses (GI and GII) showed negative correlation with pig-specific *Bacteroides* marker (r=-0.173; P<0.05), and did not show significant correlations with poultry- and ruminant-specific *Bacteroides* markers. Also, both coliforms were not correlated significantly with human-specific *Bacteroides* marker.

I									
Types	Human-specific Bacteroides marker	Poultry-specific Bacteroides marker E	Pig-specific 3acteroides marke	Ruminant-specific r <i>Bacteroides</i> marker	Norovirus GI	Norovirus GII	Norovirus GI+GII	Total coliforms	Fecal coliforms
Human-specific Bacteroides marker	1.000								
Poultry-specific Bacteroides marker	0.259** ^a	1.000							
Pig-specific Bacteroides marker	0.184*	0.132	1.000						
Ruminant-specific Bacteroides marker	0.125	0.069	0.192*	1.000					
Norovirus GI	0.263**	-0.059	-0.135	-0.046	1.000				
Norovirus GII	0.374***	0.010	-0.196*	-0.061	0.877***	1.000			
Norovirus GI+GII	0.337***	-0.019	-0.173*	-0.067	0.934***	0.969***	1.000		
Total coliforms	0.078	-0.112	-0.028	-0.035	0.299**	0.296**	0.304 **	1.000	
Fecal coliforms	0.124	-0.128	0.050	-0.032	0.0236**	0.214*	0.233**	0.835***	1.000
Asterisks indicate	statistical significan	ce (*:P<0.05; **:P<0.	01; ***.P<0.001)						

Table 4. Spearman correlation coefficients among *Bacteroides* markers, noroviruses, and coliforms in surface water samples

32

IV. Discussion

All water samples showed more than 10% occurrence of human-, poultry-, and pig-specific *Bacteroides* markers, indicated that *Bacteroides* spp. markers were widely presented in the studied areas and *Bacteroides*-specific genetic markers could exhibit stable persistence, which is appropriate to measure.

The mean concentration and occurrence of poultry- and pig-specific *Bacteroides* markers in surface water samples from Aphae Island were higher than those in surface water samples from the Goseong Bay (Table 2), which may be affected by the number of animals (31, 34). But interestingly, the mean concentration of human-specific *Bacteroides* marker in surface and seawater samples from Aphae Island was significantly higher than that in water samples from the Goseong Bay (P < 0.05), even though the residential population in the Goseong Bay are much higher than the population of Aphae Island. It may be explained by public sewage treatment connect rates, which are higher in the Goseong Bay (85.5%) than in Aphae Island (19.6%) (32). As population unconnected to public sewage treatment including the use of septic tanks directly or indirectly affect the aquatic environment (45),

public sewage treatment connect rates could be involved in the mean concentration of human-specific *Bacteroides* marker in both areas. Thus, public sewage treatment should be considered important to prevent fecal contamination in the water environments (46).

Generally, the higher mean concentration of host-specific *Bacteroides* markers were detected intensively from Autumn to early winter (September to December 2015) regardless of the geographical condition and water type (Figs. 2 and 3). Temperature is one of the major factors affecting the persistence of Bacteroides spp. in environment (47). Previous studies reported that Bacteroidesoriginated genetic markers showed longer persistence at low temperature in surface water and seawater through its prevention effects to *Bacteroides* 16S rRNA gene decay (48, 49). Because of the possibility of overestimating or underestimating the effects of influx of fecal contaminants, temperature effect should be considered for microbial source tracking with host-specific *Bacteroides* markers (50). Additionally, further longitudinal researches for our study areas are necessary to reveal the effects of various environmental factors.

The concentration of host-specific Bacteroides markers-combined

geospatial analysis revealed that the definite upstream to downdtream tendencies of fecal contamination (Figs. 4 and 5). These results clearly indicated that the fecal contaminants in surface water could be one of the major contaminants of seawater. Moreover, concentration of hostspecific *Bacteroides* markers varied according to land use composition (51). Previous studies have shown that the concentration of fecal indicators was high in waterbodies within residential areas (51, 52). In agreement with previous researches, the higher mean concentration of human-specific *Bacteroides* marker was shown in the Site AUb1 and Site AUb2 from Aphae Island, and Site GUb3 and Site GUd2 from the Goseong Bay, located near densely populated residential areas. Interestingly, the mean concentration of poultry- and pig-specific Bacteroides markers was also higher in residential areas. It may be explained by the characteristics of the areas where small-scale livestock breeding occurred in residential areas. In addition, in agricultural sites, the high concentration of Bacteroides markers was observed because of fertilzers contained human or animal feces (53, 54). Therefore, our study demonstrated that the host-specific Bacteroides markerscombined geospatial analysis could be the useful tool for predicting environmental effects of potential fecal sources.

Previous studies have investigated the correlation between microbial indicators and human enteric viruses in natural aquatic environment (27, 55). McQuaig et al. (27) reported the presence of human adenoviruses was correlated with human-associated *Bacteroides* species. Also, Hughes et al. (55) showed that human-related *Bacteroides* was correlated with human polyomavirus and norovirus. In agreement with previous researches, our data demonstrated human-specific *Bacteroides* marker was significantly correlated with human noroviruses have a common characteristic of strong human-specificity (25), human-specific *Bacteroides* marker could be useful to trace the waterborne transmission of human noroviruses specified with the origin of viruses-contaminated feces.

In conclusion, the host-specific *Bacteroides* markers used in this study are suitable genetic markers for fecal source tracking regardless the geographical differences, such as open-island form (Aphae Island) and closed-bay form (the Goseong Bay). Despite their seasonal tendencies of concentration and occurrence, host-specific *Bacteroides* markers combined with geospatial analysis showed the clear transition of fecal contamination. In addition, human-specific *Bacteroides* marker showed a significant correlation with human noroviruses. Therefore, host-specific *Bacteroides* markers with advanced geospatial analyses could be a useful tool for tracking fecal contamination in water environment.

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국문초록

숙주 특이적 *Bacteroides* 종 기반 국내 미생물 오염원 추적 연구

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44

다양한 수인성 병원균은 인간과 동물의 분변에서 유래하며, 위장관염의 주요한 원 인이다. Bacteroides는 강한 숙주 특이성을 가지기에 분변 오염원을 보다 면밀히 구분할 수 있어, 미생물 오염원 추적 연구에 적합한 지표로 알려져 있다. 본 연구 는 지리적 차이를 보이는 압해도 및 고성만의 수시료에서 4 종류의 숙주 특이적 Bacteroides (인간, 가금류, 돼지, 반추동물)의 유전자를 정량하여, 분변 오염을 평 가하고자 하였다. 아울러 Bacteroides 마커-지리정보 분석을 통하여 분변 오염원 을 예측하였으며, 인간 특이적 Bacteroides 마커와 인간 노로바이러스 사이의 상 관성 역시 분석하였다. 숙주 특이적 Bacteroides 마커는 연구지역 전반에 걸쳐 넓 게 분포되어 있었으며, 특히 가금류 특이적 Bacteroides 마커는 압해도에서 1.2 log10 copies/L, 고성만에서 1.0 log10 copies/L 등으로 가장 높은 농도를 보였다. 또한 2015년 9월-12월 중 숙주 특이적 Bacteroides 마커는 타 시료채취기간에 비해 상대적으로 높은 농도를 보였는데, 해당 기간 동안의 낮은 수온에 의해 Bacteroides 마커의 농도가 높아졌을 것이라 추측되었다. Bacteroides 마커-지리 정보 지도를 통해, 숙주 특이적 Bacteroides의 농도가 상류에서 하류로 갈수록 감 소하는 경향을 확인하였으며, 토지 이용 현황에 따른 마커의 농도 차이 역시 확인 하였다. 또한 인간 특이적 Bacteroides 마커는 총대장균군과 분원성대장균군과 같 은 전통적인 지표 미생물에 비하여, 인간 노로바이러스와 보다 높은 상관관계를 나 타내었다. 이에 숙주특이적 Bacteroides 마커는 발전된 지리정보 분석과 더불어서 활용할 경우 수 환경 중의 분변 오염 추적에 있어 적합한 도구로 활용할 수 있을 것이다.

주요 단어: 분변 오염, 미생물 추적 연구, 숙주특이적 박테로이데스 마커, 인간 노 로바이러스, 지리 정보 시스템

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