

Epidemiological Study of Outbreak of Gastroenteritis Associated with Norovirus and Astrovirus in Busan, Korea

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This paper studies an infection of norovirus and astrovirus in outbreaks in Korea. In March 2016, gastroenteritis outbreaks occurred in Busan. 522 students of three departments at university D had meeting at a restaurant near the university. Some of them had symptom such as diarrhea, vomiting. Epidemiological, laboratory and environmental investigations were performed to identify the agents of the outbreaks. Fecal specimens were collected from 35 students and 7 food handlers to identify causative viral agents. Norovirus genogroup GI and GII were detected from diarrhea patients. Astrovirus was also detected from some of them. In particular, these outbreaks were the first occurrence associated with astrovirus in Busan. Total of 42 samples were collected, and 24 samples resulted in positive to norovirus (16 cases) and astrovirus (8 cases). To identify the molecular genetic information of norovirus, we carried out sequences analysis of the detected strains. Norovirus genotypes were classified into GI.3, GI.4, GII.4, GII.13, GII.17 and GII.21. Astrovirus genotypes were seven astrovirus type 5 and one astrovirus type 2. We performed environmental investigation about water at the kitchen, but norovirus and astrovirus were not detected. The statistical analysis was conducted to evaluate the association between illness and risk factors. The results of this study may contribute to accumulate more the epidemiological data and develop the public health and hygiene.

Key words : Astrovirus, molecular epidemiology, outbreaks

Introduction

Acute gastroenteritis has been known as one of the most frequent diseases and became a major public health issue worldwide. The surveillance of gastroenteritis in Korea has revealed that gastroenteritis caused by viruses becomes more common and viral infections increase [7]. Now norovirus becomes a major causative agent of gastroenteritis outbreaks reported worldwide. Norovirus is a non-enveloped, single-stranded RNA virus, classified into five genogroups, so called GI~GV, based on amino acid identities in the VP1 (ORF2) [5]. According to a previous study [13], norovirus can be classified into genotypes, with 9 genotypes belonging to genogroup GI and 22 genotypes of genogroup GII, which is used as the classification standard in this paper. In recent decades, norovirus GII.4 has been reported as the

most common circulating genotype worldwide [8]. Recently, norovirus GII.17 is becoming the remarkable type causing large-scale outbreaks of gastroenteritis [14]. The symptom of norovirus is characterized by acute onset, nonbloody diarrhea, vomiting, nausea and abdominal pains.

Astrovirus is a causative enteric pathogens, and it can be classified genetically into eight genotypes based on the capsid protein precursor (ORF2) [17]. Astrovirus type 1 has been reported as a predominant type in the world. Astrovirus is a small, non-enveloped, round, single-stranded RNA virus [15]. Astrovirus induces a watery mild diarrhea that is lasting during 2~3 days and may be related to vomiting, fever and abdominal cramp [16]. However, astrovirus is clinically less severe than norovirus infections [3, 21].

There are some researches about astrovirus outbreaks in various countries. Astrovirus infection was very common in children more than 3 years old in France [2]. 80% of astrovirus infections occurred in children less than 3 years old in Spanish [6]. In contrast, there was an outbreak of astrovirus gastroenteritis in residential care home for elder people in Scotland [9]. In Korea, the outbreak of the astrovirus infection was occurred in riot police camp in 2014 [8]. Astrovirus may be a major cause of gastroenteritis in

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young children, elderly people and immunocompromised adults [18]. The public health authority needs to give more close attention to astrovirus because of the outbreaks in adults [1, 19].

This study conducts an epidemiological analysis on the outbreaks incidence of acute gastroenteritis of the university students in March 2016, Busan. We collected data associated with the outbreak information including dates, locations, number of patients, patient symptoms and contributing factors. We identified the epidemic characteristics of norovirus and astrovirus from the outbreaks. In this study, we report firstly that astrovirus was the causative agent to the acute gastroenteritis outbreaks in Busan until now.

Materials and Methods

Epidemiological investigation

On March in 2016, some students of department A in university D attended a meeting at a restaurant, and some patients with diarrhea, vomiting, chills visited the public health center. The epidemiological investigation team recognized that there were similar cases of department B and department C in the university. All outbreaks occurred at the same restaurant. The epidemiological investigation team had a site survey, and obtained the menu of the restaurant, and investigated other external factors. The restaurant used the water supplied by mixing tap water and underground water. We collected the water 1.8 l by using the water sampler at the kitchen in the restaurant.

Specimens and RNA extraction

Fecal specimens were collected from 35 acute gastroenteritis patients and 7 food handlers. The specimen was diluted in phosphate buffered saline and vortexed for 3 min,

centrifuged at 3,000 rpm, for 20 min, and collected the supernatant. Viral RNA was extracted using a GM-autoprep Kit (Green Mate, Korea) according to the manufacturer's instructions. The environmental sample was tested according to the Food code of the ministry of food and drug safety.

The detection and genotyping of norovirus and astrovirus

Norovirus detection was conducted using an Real-time reverse transcriptase-polymerase chain reaction (Real-time RT-PCR) and RT-PCR using Real-time RT-PCR Kit (Bioneer, Korea) with the following conditions: reverse transcription at 45°C for 30 min, pre-denaturation at 95°C for 10 min, 45 cycles of denaturation at 95°C for 15 sec, annealing and extension at 56°C for 1 min. RT-PCR and Semi-nested RT-PCR were performed to identify genotypes. Astrovirus was detected by RT-PCR using a one-step premix kit (SNC, Korea) under the followed conditions: 48°C, 40 min; 94°C, 15 min; 94°C, 30 sec; 58°C, 30 sec; 72°C, 60 sec for 35 cycles; and 72°C 7 min. The primers of oligo nucleotide sequence for detection of norovirus, astrovirus are shown in Table 1.

Nucleotide sequencing and phylogenetic analysis

The PCR products were purified using a QIAquick Gel Extraction kit (Qiagen, Germany). Sequencing analysis was performed using a Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in ABI 3730XL Genetic Analyzer (Applied Biosystems, USA). The partial sequences of the capsid genes of norovirus and astrovirus isolates were aligned by Cluster W and compared to reference strains (Table 2). The phylogenetic analysis was determined using MEGA program based on the neighbor-joining method with 1,000 bootstrap replicates for genotype classification.

Table 1. The primers used for the detection of NoV and AstV

Virus	Primer	Sequences (5'-3')	Size (bp)	Application
NoV ¹⁾ GI	GI-F1M	CTGCCCGAATTYGTAAATGATGAT	314	Onestep RT-PC Onestep RT-PC/Semi-nested PCR Semi-nested PCR
	GI-R1M	CCAACCCARCCATTTRTACATYTG		
	GI-F2	ATGATGATGGCGTCTAAGGACGC		
NoV ¹⁾ GII	GII-F1M	GGGAGGGCGATCGCAATCT	313	Onestep RT-PC Onestep RT-PC/Semi-nested PCR Semi-nested PCR
	GII-R1M	CCRCCIGCATRICCRITRTACAT		
	GII-F3	TTGTGAATGAAGATGGCGTTCGART		
AstV ²⁾	MON269	CAACTCAGGAAACAGGGTGT	449	Onestep RT-PC
	MON270	TCAGATGCATTGTCATTGGT		

¹⁾NoV : Norovirus, ²⁾AstV : Astrovirus

Table 2. Norovirus reference strains (GenBank accession No.) used for sequencing analysis

NoV GI	GenBank accession No.	NoV GII	GenBank accession No.	AstV	GenBank accession No.
GI.1	L23828, M87661	GII.1	AJ277606, U07611	Type 1	AY720892-Dresden
GI.2	AJ277610, L07418	GII.2	AY134748, X81879		L23513-Oxford
GI.3	AB187514, AJ277612	GII.3	EU187437, U02030	Type 2	L13745-Oxford
	AY038598, EF547396	GII.4	X76716	Type 3	AF141381
	GQ856470, GQ856471	GII.5	2007JP-OB200615		DQ630763-WH1859
GI.4	GQ856473, U04469	GII.6	AF39715, AJ277607	Type 4	DQ070852-Brazil
	AB042808, AJ277616	GII.7	AB03977, AJ277620		AY720891-Dresden
GI.5	AJ277621, AJ313030	GII.8	AF41440, AJ277608		DQ344027-Guangzhou
	AB039774, AF414406	GII.9	AB039780, AF195848	Type 5	DQ028633-Brazil
GI.6	AJ277614, AM263418	GII.10	AY038599, DQ379715	Type 6	AB013618-Osaka
	AF093797, AF538678	GII.11	AF427118, AY237415	Type 7	Y08632
GI.7	AJ277615	GII.12	AB074893, AB126320	Type 8	AF260508-Yuc
	AJ277609, AJ844469	GII.13	AB032758, AJ277618		
GI.8	AY675555	GII.14	AB078334, AY113106		
	AF538679, GU299761	GII.15	AY130761, GQ856465		
GI.9	GU296356, HQ637267	GII.16	AY130762		
		GII.17	AY502010, GQ856476		
		GII.18	AY502009, DQ438972		
		GII.19	AB983218-Kawasaki		
		GII.20	AY823304, AY823305		
		GII.21	AY823306, AY823307		
		GII.22	AB542917, EU373815		
			AB542915, AY675554		
			AB083780, GQ856469		

Statistical analysis

We conducted statistical analysis such as odds ratio test, attributable risk test and the chi-square test to analyze the characteristics of the infection trends by using SPSS software. From the statistical results, we can conclude that the associated food with *p*-value (<0.05) is considered significantly associated with illness.

Results

Epidemic distribution of the outbreaks

Cases were defined as patients with at least two symptoms within 24 hours. The number of patients was 15 of group A, 16 of group B, and 4 of group C. The infection rates are 12.3%, 5.0%, and 5.0% respectively, which is presented as in Table 3.

The first patients in group A with symptoms of diarrhea, abdominal pain, nausea and fever appeared on March 11 and the number of patients peaked on March 12. The disease onset of group B was on March 12 and the number of cases peaked on March 13. By the way, illness onset of group C

Table 3. The summary of the outbreaks

Visit date	2016.3.10	2016.3.11	2016.3.15	Total
Group	Group A	Group B	Group C	
Exposed persons	122	320	80	522
Patients	15	16	4	35
Positive rates	12.3%	5.0%	5.0%	6.7%

was on March 17 and number of peaked case on same date (Fig. 1).

The symptoms of the outbreaks were diarrhea, nausea, abdominal pain, vomiting, chills, fever, headache. All the patients from groups A, B, C had mainly diarrhea, and patients had abdominal pain 77.1% and nausea 71.4%, which can be observed as in Table 4.

Laboratory analysis

For fecal specimens and environmental sample, the laboratory tests were performed and shown in Table 5. Among 35 samples from patients, 16 patients were norovirus positive and 8 patients were astrovirus positive. For norovirus

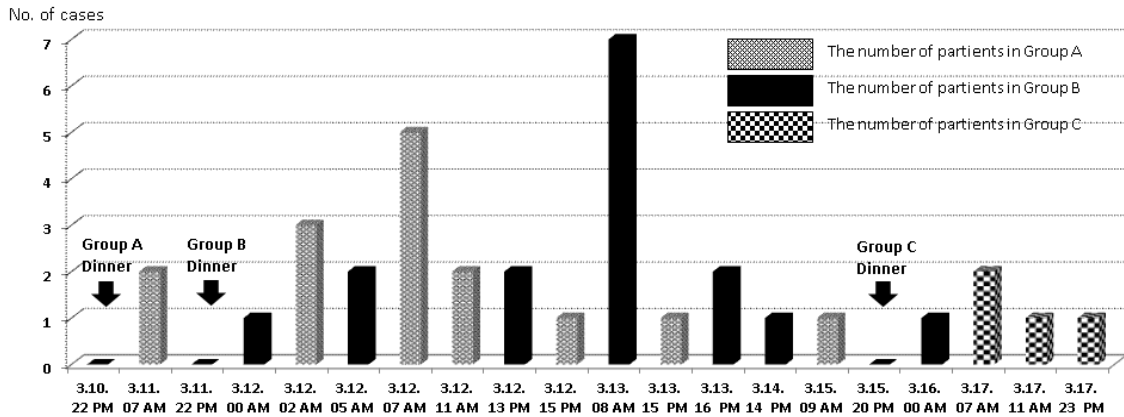


Fig. 1. The number of patients on each date of groups A, B, C.

Table 4. Symptom-wise distribution of patients

Symptom	No. of patients			
	Group A (n=15)	Group B (n=16)	Group C (n=4)	Total (n=35)
Diarrhea	15	16	4	35 (100%)
Fever	4	5	2	11 (31.4%)
Chills	7	7	2	16 (45.7%)
Nausea	11	11	3	25 (71.4%)
Vomiting	6	10	3	19 (25.7%)
Abdominal pain	14	10	3	27 (77.1%)
Headache	3	1	1	5 (14.3%)
other	1	1	0	2 (5.7%)

detection, 53.3% were norovirus positive in group A, 31.3% in group B, 75% in group C. For astrovirus detection in each

group, there was no astrovirus positive in group A, but, 37.5% in group B and 50% in group C.

The distributed genotypes of norovirus and astrovirus are presented in Table 6. Norovirus genotypes were GI.3, GI.4, GII.4, GII.13, GII.17 and GII.21. Norovirus GII.17 was mostly detected among all genotypes (43.8%, 7/16). The genotype of astrovirus was identified as type 5 in seven patients and type 2 in only one patient. There are three patients infected with norovirus and astrovirus simultaneously. The first patient was infected with GI.3 and astrovirus type 5, and the second patient with GI.4 and astrovirus type 5, the last patient with GII.21 and astrovirus type 5.

Phylogenetic analysis of the astrovirus and norovirus was performed based on the primers and reference stains in

Table 5. The detected viruses related to outbreaks in Busan

	Group A (n=15)	Group B (n=16)	Group C (n=4)	Total (n=35)
No. of Norovirus detection	8 (53.3%)	5 (31.3%)	3 (75%)	16 (45.7%)
No. of Astrovirus detection	0	6 (37.5%)	2 (50%)	8 (22.6%)

Table 6. The genotypes of distribution cases

Virus	Genotypes	No. of Norovirus GI	No. of Norovirus GII	No. of Astrovirus	Total
Norovirus	GI.3	2 ^{*)}			2 ^{*)}
	GI.4	1 ^{*)}			1 ^{*)}
	GII.4		1		1
	GII.13		2		2
	GII.17		7		7
	GII.21		3 ^{*)}		
Astrovirus	type 2			1	1
	type 5			7 ^{**)}	7 ^{**)}
Total		3	13	8	24

^{*)} One of figure is co infection with astrovirus.

^{**)} Three of figure are co infection with norovirus.

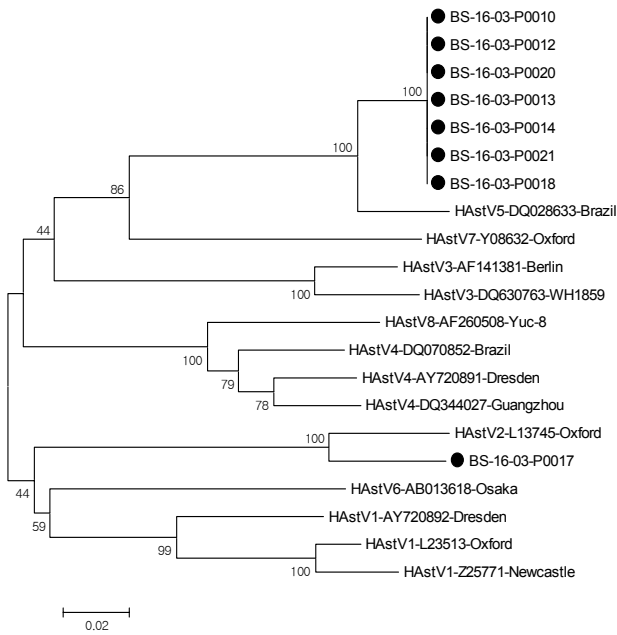


Fig. 2. Phylogenetic analysis of the astrovirus and strains from patients are indicated by the shaded black circles.

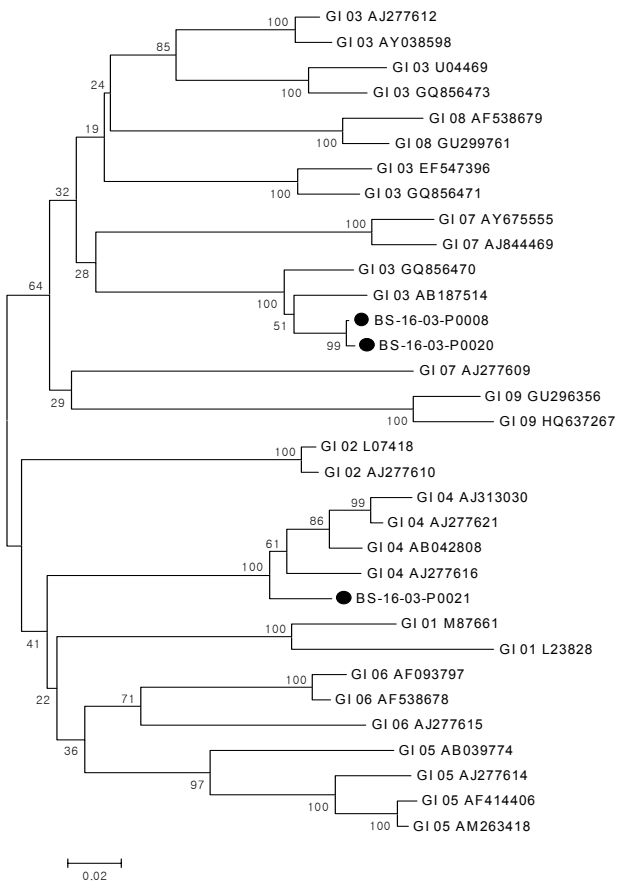


Fig. 3. Phylogenetic analysis of identified norovirus based on GI and strains from patients are indicated by the shaded black circles.

Table 1, 2. Phylogenetic analysis of astrovirus revealed that the detected type 5 strains tend to be closely related to each other and are classified into Brazil strains. Thus, it

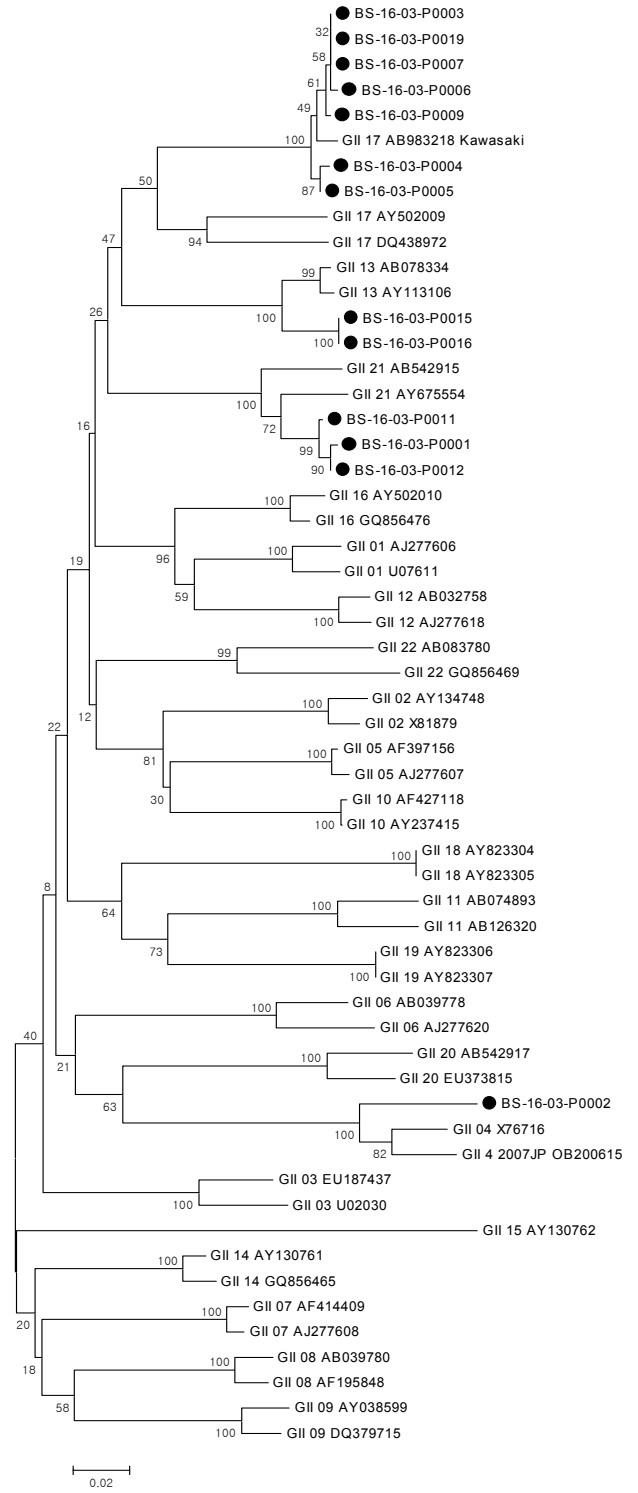


Fig. 4. Phylogenetic analysis of identified norovirus based on GII and strains from patients are indicated by the shaded black circles.

is observed in Fig. 2 that the similarities of the nucleotide sequences is 100% for AstV type 5. Phylogenetic trees of norovirus are shown in Fig. 3 (GI) and Fig. 4 (GII). The genotype of sequences was decided in agreement with more than 90 % identity of reference sequences. The nucleotide sequence previously registered in the GenBank also had a high level of similarity at 94.8-96.0% with GI and 97.0-98.6% with GII.

Note that the dates of the outbreaks at the restaurant are various and there is no mandatory regulation that the private restaurant keeps the food ingredients unlike school cafeterias. Thus it is difficult to identify directly the pathogen on food because the food and ingredients don't remain at the restaurant. Moreover there was no norovirus and astrovirus positive in the underground water. We investigated the food which patients had on that date, which are summarized as in Table 7. This study conducted the odds ratio and the chi-square test to analyze statistically the characteristics of the infection causes. Through the statistical methods, the

odds ratio values with 95% confidence intervals (CI) and the *p*-values are obtained. It can be observed from Table 7 that chicken and filtered water (on March 10), sweet and sour pork, fish cake soup and filtered water (on March 11) have *p*-value<0.05 and the high odds ratio values. Based on these results, we can remark that the foods with *p*-value<0.05 seem to be significantly associated with illness.

Discussion

The outbreaks occurred among the university students which are 20's healthy people. The causative viral agents of the outbreaks were indicated as norovirus, astrovirus and co-infection of them. A total of 16 norovirus strains were sequenced and clustered into two distinct genogroups, GI and GII. Six kinds of genotypes were found as GI.3, GI.4 GII.4, GII.13, GII.17 and GII.21. In particular, GII.17 become the leading cause of norovirus related gastroenteritis in these

Table 7. Association of illness with foods

Group Date	Meal	No. of patients			No. of controls			<i>p</i> -value	Odds ratio (95% CI*)	Attributable risk
		intake	No intake	Total	intake	No intake	Total			
Group A	Sweet and sour pork	11	4	15	21	9	30	0.9074	1.1786	0.0360
	Fish cake soup	8	7	15	18	12	30	0.9150	0.7619	-0.0607
	Assorted sausages	2	13	15	3	27	30	0.8668	1.3846	0.0750
	Assorted fried	0	15	15	0	30	30	-	-	-
	Chicken	12	3	15	11	19	30	0.0153	6.9091	0.3853
	Spicy Sausage Stew	1	14	15	6	24	30	0.4672	0.2857	-0.2255
	Tricolored cookies	2	13	15	3	27	30	0.8668	1.3846	0.0750
	Filtered water	13	2	15	6	24	30	0.0001	26.0000	0.6072
Group B	Sweet and sour pork	14	2	16	10	24	34	0.0004	16.8000	0.5064
	Fish cake soup	14	2	16	12	22	34	0.0017	12.8333	0.4551
	Assorted sausages	2	14	16	1	33	34	0.4906	4.7143	0.3687
	Assorted fried	1	15	16	0	34	34	0.6967	-	-
	Chicken	5	11	16	13	21	34	0.8696	0.7343	-0.0659
	Spicy sausage stew	2	14	16	1	33	34	0.4906	4.7143	0.3687
	Tricolored cookies	3	13	16	2	32	34	0.3631	3.6923	0.3111
	Filtered water	15	1	16	9	25	34	0.0000	41.6667	0.5865
Group C	Sweet and sour pork	2	2	4	5	5	10	0.5541	1.0000	0.0000
	Fish cake soup	3	1	4	5	5	10	0.7978	3.0000	0.2083
	Assorted sausages	0	4	4	0	10	10	-	-	-
	Assorted fried	0	4	4	0	10	10	-	-	-
	Chicken	2	2	4	4	6	10	0.7978	1.5000	0.0833
	Spicy sausage stew	1	3	4	1	9	10	0.9039	3.0000	0.2500
	Kimchi stew	0	4	4	0	10	10	-	-	-
	Duruchigi	0	4	4	1	9	10	0.6225	0.0000	-0.3076
	Tricolored cookies	0	4	4	3	7	10	0.6066	0.0000	-0.3636
Filtered water	3	1	4	2	7	9	0.2350	10.5000	0.4750	

*CI : confidence interval

outbreaks. Norovirus GII.17 emerged in 2014 as the predominant genotype of causing acute gastroenteritis in China. Some researchers forecast that norovirus GII.17 will cause the next global epidemic [4, 12, 20], and suggests to track the outbreaks caused by this strain globally. Thus, it is necessary to continue monitoring epidemiological trends of norovirus surveillance in its geographical spread and evolution.

In addition, astrovirus has been distributed in the world and prominent in Asia and Europe. Astrovirus type 1 is a predominant strain in Korea and worldwide. Then, astrovirus type 1 may have almost immunized many people and therefore is not infectious anymore [10]. By contrast, astrovirus type 5 is an uncommon strain in Korea, and a few individuals possess immunity. In this study, 8 astrovirus strains were detected and 7 of them were identified astrovirus type 5. Astrovirus generally induces mild diarrhea in infants [2, 6, 11], however in this study, astrovirus infection induced gastroenteritis especially in adults. This paper found that norovirus GII.17 and astrovirus type 5 were the most predominant genotypes. Therefore, it can be concluded that the genotypes identified in this study might be the significant types in outbreaks in Korea.

However, norovirus and astrovirus were not detected in ground water at the kitchen of the restaurant. Indeed, the statistical risk showed the exposure to patients and sharing a filtered water was associated with the spread of infection. The analyzed results suggest that there may be several infection routes for the norovirus and astrovirus infection.

This is the first report of molecular epidemiology of norovirus and astrovirus infections in acute gastroenteritis, associated with outbreaks in Busan, Korea. The outbreaks related to astrovirus have not been found in Busan before, and has been rarely studied in the literature. Consequently, this paper considered an important epidemic outbreak. In addition, the genetic characteristics of norovirus and astrovirus from patients have also been analyzed, and it was characterized statistically the transmission route that might cause gastroenteritis outbreaks.

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초록 : 집단식중독 환자에서 검출된 노로바이러스 및 아스트로바이러스의 분자역학적 연구

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2016년 3월, 부산 D대학 인근 음식점에서 모임을 한 후, 설사, 구토를 호소하는 환자들이 발생하였다. 역학조사팀은 설사 환자 및 해당 음식점 조리종사자에 대한 분변 검체를 수집하였고 해당 음식점 주방에서 식품용수에 대한 채수도 진행하였다. 인체 검체 42건에서 노로바이러스 16건, 아스트로바이러스 8건이 검출되었으며, 노로바이러스의 경우는 GI, GII genogroup 모두 검출되었으며, GI.3, GI.4, GII.4, GII.13, GII.17, GII.21로 6가지 다양한 유전자형의 분포를 확인하였다. 아스트로바이러스의 경우 Type 5과 Type 2의 유전자형 분포양상을 확인하였다. 또한 노로바이러스와 아스트로바이러스가 복합 감염된 3 케이스도 포함되어 있었다. 노로바이러스는 전세계적으로 GII.4형이 유행하고 있고, 최근에는 GII.17형이 출현하고 급증하는 동향에 따라 본 연구에서도 GII.17형이 가장 우세하였으며, 아스트로바이러스 경우는 국내에서 우세한 유전자형인 Type 1인 것과는 차이가 있는 사례였다. 특히, 부산지역에서 아스트로바이러스가 식중독 발생의 원인이 된 경우는 이번이 첫 사례여서 본 연구를 통하여 부산지역의 식중독 발생에 새로운 발생 양상을 파악하는 매우 특징적 결과를 얻었다.