# Detection of Enteric viruses from urban river water in the Philippines

# Detection of Enteric viruses from urban river water in the Philippines

**T. Imagawa[1], A. Suzuki[1], M. Saito[2], Y. Masago[3], C. Okumura[3], S. Lupisan[4], R. Olveda[4], T. Omura[3], and H. Oshitani[1]**

**[1] Tohoku University Graduate School of Medicine, Department of Virology, [2] Research Center for Emerging and Re-emerging Infections, Manila, [3] Tohoku University, Department of Civil and Environmental Engineering, [4] Research Institute for Tropical Medicine, Manila**

## Abstract

Diarrheal diseases are one of the leading causes of childhood morbidity and mortality in developing countries. In the Philippines, approximately 1,000 infants die of diarrhea annually. Since enteric viruses shed in feces of diarrheal patients will be drained into sewage, environmental water can be at risk of the viral contamination. Therefore we tried to quantify enteric viruses in river water of the Philippines to assess the risk of acquiring viral infection from environmental water. We collected water from rivers in Metro Manila and Bulacan (north of Manila) in dry and wet season. We concentrated water samples by poly-ethylene glycol precipitation method and detected adenoviruses, enteroviruses, hepatitis A virus, norovirus GI and GII and group A rotavirus by quantitative real time PCR, and astrovirus by conventional PCR. We found that the river water was highly contaminated with feces and had high virus titer compare to the river in other countries. Some enteric viruses had higher viral titer in dry season, and viral titer in water depended on the population density in the catchment area. Our sequence analysis showed that detected rotavirus and hepatitis A virus were not specific for the Philippines but common genotypes which had been circulating among human in other countries. We concluded that there is risk of enteric virus infection from urban river water in the Philippines.

**Key words**: the Philippines, river water, enteric virus, quantification, phylogenic analysis

## Introduction

Diarrhea is one of the major health problems, and about 1.8 million children die of diarrhea annually (Cunliffe et al. 2005). In developing country where clinical care is not adequate, diarrhea can be life-threatening disease.

The major causes of diarrhea appear to be different significantly between developed and developing countries. In the developed countries, the most common causes of diarrhea are viruses, and norovirus and rotavirus are mainly detected from diarrhea patients (Nakanishi et al. 2009). On the other hand in developing countries, bacterial agents have been reported to be accounted for most diarrheal diseases (Fereshteh et al. 2008). This difference may be due to detection bias since virological tests are usually not available in most of developing countries.

Even in developed country, enteric viruses can be found in environmental water (Yamamoto et al. 2005, Yano 2006). Moreover, where water treatment for sewage is not be adequate, water can be the source of infection since viruses may go into a cycle between human and environment. For examples, norovirus shed in feces will be drained into sewage water to river and sea, and the virus may be concentrated in the mid-gut of shellfish, and that can become a source of infection in human as a food-borne disease. In some developing countries, untreated river water is used for their daily life such as drinking, washing, and leisure. If water were to be contaminated by pathogenic microorganisms, people in those areas are exposed to them through their daily life. Safe water supply may decrease morbidity and mortality of diarrheal disease. There is a need to access the risk of acquiring not only the bacteria but also enteric virus infection of river water.

In the Philippines, Research Institute for Tropical Medicine (RITM) under

Department of Health is a national reference center for diarrheal disease of the Philippines. According to the data from RITM, enterotoxigenic *Escherichia coli* (*E.coli*), *Vibrio cholera* and salmonella have been main pathogens detected from outbreaks in the community. For enteric viruses, only rotavirus had been documented (Marilla et al. 1984; Celia et al. 1990). There is limited available data for other enteric viruses in the Philippines.

Pasig River is the one of the river that runs through the Metro Manila. Kato et al had shown that Pasig river had high biochemical oxygen demand (BOD) level which implies that the river was highly polluted with organic matter such as feces (Kato 1999). On the other hand, there is no data on enteric viruses in the environment water in the Philippines.

Since there is limited data on the epidemiology of the enteric viruses in the Philippines, we have chosen common enteric viruses based on the study conducted in other counties. The following viruses were included in the study;

1) Adenovirus belongs to *Adenoviradae*. Viral particle is about 100 nm diameters. It does not have envelope which is determinant of the stability. If envelope is destroyed, then virus loses infectivity. It has double strand DNA as genome. DNA is much more stable than RNA in natural environment where RNAase exists everywhere. There are 51 serotypes for human adenovirus and around a third of these serotypes are pathogenic for human. Adenovirus 40 and 41 are well known cause of viral diarrhea.

2) Enterovirus belongs to family *Picornaviradae*. Enterovirus consists of coxakie virus A, B, echovirus, poliovirus, and enterovirus. These viruses have single strand positive sense RNA as genome, but does not have envelope. Enterovirus causes

variety of symptoms such as diarrhea, meningitis, herpangina and Hand-Foot-Mouth disease. In Japan, enteroviruses are mainly circulating in summer in temperate climate.

3) Hepatitis A virus (HAV) also belongs to *Picornaviradae*. HAV has only one serotype but 9 genotypes, and genotype IA is the most common type of HAV all over the world. In developing country, most of people have antibody for this virus since they are exposed to HAV when they are young. In the Philippines, almost 100% of the residents of a poor rural community had anti-HAV antibodies by the age of 15 years (Domingo EC. 1986). On the other hand, a seroprevalence in the middle-income/middle-class communities in Manila found that only 48% were antibody-positive at a similar age (Bazga NG. 2000). There must be socioeconomic factor that relates to the exposure to the HAV.

4) Norovirus belongs to *Caliciviridae*, norovirus genus. It has single strand positive sense RNA as genome. Norovirus has 5 genogroups from GI to GV. GI, II, and IV can infect to human, and GI and II are most frequently detected from human. This virus causes mainly diarrhea with vomiting. Norovirus is one of the most common viruses causing gastroenteritis in developed countries. Recently, norovirus GII/4 is a predominant strain circulating worldwide, including China (Jin et al. 2009), USA (Roger et al. 2009) and Japan (Okada et al. 2005). Norovirus is frequently detected from environmental water and shellfish (Yamamoto et al. 2005; Yano 2006; Tiong et al. 2009).

5) Rotavirus belongs to *Reoviradae* rotavirus genus. The virus has 11 segments of double strand RNA as viral genome. The outer capsid layer is composed of two proteins; VP defines G types and VP4 defines P types. At least 15 G types and 27 P

types have been found by sequence analysis of the VP7 and VP4. The virus has 7 groups from A to G, but only groups A, B, and C rotavirus cause gastroenteritis to human. Most common rotavirus is group A rotavirus, and it often causes severe diarrhea with dehydration in infants. Usually, this virus spread in winter season in temperate country. Rotavirus is as common as norovirus in diarrheal patients in developed countries. In the Philippines, rotavirus was more common than bacteria in diarrheal patients (Marilla et al. 1984).

6) Astrovirus belongs to family *Astroviridae*, mamastrovirus genus. It has single strand positive sense RNA as viral genome, but does not have envelope. Human astrovirus has 8 serotypes, G1 to G8, and G1 is the major circulating serotype. Most of people have antibody for this virus and its symptom is not so severe. Astrovirus is frequently detected from diarrheal patients in many countries, including Taiwan (Hsiao-Chuan et al. 2008), Japan (Nakanishi et al. 2009), Pakistan (Tung et al. 2004), and Vietnam (Tuan et al. 2007). Serologic surveys in the UK and USA have shown that more than 70% of children acquire astrovirus infection by 5 years of age (Kultz et al. 1978).

We conducted this study to access the prevalence of the enteric viruses in the river water to anticipate the risk of viral infection due to contamination of environmental water. We expect that this study provide useful data for public health in the Philippines.

## Materials and Methods

*Water sampling sites*

We selected 6 sites in Metro Manila and 8 sites in Bulacan in Luzon island, the Philippines (Figure1a).

*1) Metro Manila region*

Metro Manila is located in National Capital Region of the Philippines. The population of Metro Manila is 11,553,427 at 2007 (National Statistics Office), area is 638.55 $km^2$ and population density is 18,246 people/$km^2$. We have selected Las Piñas and Parañaque as sampling sites as shown in Figure1(b). Las Piñas sites (La1, 2) are located in tributary of Las Piñas river and Parañaque (Pa1-4) sites are located in tributary of Parañaque river.

*2) Bulacan*

Bulacan belongs to Region III of the Philippines, which is located in northern part of Metro Manila. This area is more rural and has more farm land than Metro Manila area. The population of Bulacan province is 2,826,926 in 2007 (NSO), area is 2,774.85 $km^2$ and population density is 1,077 people/$km^2$. We have chosen Meycauayan, Marilao, and Bocaue as sampling sites in Bulacan Area as showen in Figure1(c). Meycauayan sites (Me1-3) are along Meycauayan river, Marilao sites (Ma1,2) are along with Marilao river, and Bocaue sites (Bo1-3) are along with Santa Maria river.

*Sampling Time Frame*

In Western Luzon, wet season is usually from May to November and dry season from December to April based on mean precipitation data between 1971 and 2001

(Chronological Scientific Tables 2006). In 2009, annual precipitation in Metro Manila (Philippine Atmospheric, Geophysical and Astronomical Services Administration) was twice as higher than its average between 1971 and 2000 (Figure .2) and the Philippines had flood event by typhoon "Ondoy" in September 2009.We collected water samples in March, April, August, November, and in December. March survey was conducted as a pilot survey to decide sampling sites.

*Sampling Methods*

All water sampling was performed in morning. We threw a bucket from middle of the bridge over the river or from shore, and collected about 2 L of water. Then we transferred 200 ml of water from the bucket into sterilized sample bottle. They were kept on ice until further specimen processing.

*Bacteriological examination*

Bacterial tests were performed in order to assess the level of fecal contamination of river water. We collected samples from 5 lowest sites in each river (La2, Pa3, Me3, Ma2, and Bo3) in April, August, and December. We measured total coliform for *E.coli* and *enterococcus* which are generally used as an indicator of fecal contamination. Bacterial test was performed by membrane filter method (Japan Sewage Works Association). Each water sample was diluted by ten hold from 1/10 to 1/10000 with Dulbecco's phosphate buffered saline (D-PBS) (-). Five ml of sample water was filtered with 0.45 $\mu$m pore filter (HA filter: Millipore). Then the filter was put on the chromocult agar (MERCK, NJ, USA) for *E.coli* and total coliform culture, and the m-enterococcus agar (DIFCO, USA) for *enterococcus* culture.

Inoculated plates were incubate at 37 ℃ for 24 hours or 48 hours. After incubation (chromocult: 24h, m-enterococcus; 48h), we counted colonies on 3 plates for each dilution and calculated the mean number of colonies in 1ml.

*Polyethylene glycol (PEG) precipitation*

Sampled water was concentrated by polyethylene glycol (PEG) precipitation method (from "Method of Norovirus Detection" Ministry of Health, Labour and Welfare, Japan) for virus detection. PEG 6000 (Wako Pure Chemical Industries, Osaka, Japan) and sodium chloride (Wako Pure Chemical Industries, Osaka, Japan) were added to 45 ml of sample water, and the final concentrations of PEG and sodium chloride were 8 % and 2.1 %, respectively. The sample mixtures were stored at 4 ℃ over night. Then they were centrifuged at 4 ℃, 3220×g for 1 hour. The supernatant was removed and pellet was suspended with 3ml of D-PBS(-). Then suspension was filtered with 0.45um pore filter (ADVANTEC, CA, USA) and stored at -80 ℃ until viral genome extraction.

*Nucleic acid extraction and Reverse transcription*

Two hundred micro liters of the concentrated water samples were subjected to nucleic acid extraction with Purelink Viral DNA/RNA kit (Invitrogen, CA USA). Each 11 μl RNA samples were reverse transcripted with M-MLV (Invitrogen, CA, USA) using random primer (Invitrogen, CA, USA).

*Quantitative real time PCR and nested PCR*

We detected viruses with quantitative real time PCR or conventional PCR. Real

time PCR was used for adenovirus (Heim et al. 2003), enterovirus (Monpoeho et al. 2000), hepatitis A virus (Nishio et al. 2002), norovirus GI and GII (Katayama et al. 2003), and group A rotavirus (Pang et al. 2004) with 7500 Real Time PCR system (Applied Biosystems, CA, USA) using TaqMan Real time PCR master mix (Applied Biosystems, CA, USA) (Table 1). We did triplicate of real time PCR for each samples. We calculated viral concentration as PDU (PCR Detective Unit)/ml. PDU is equivalent to DNA copies. The conventional PCR was used for astrovirus (Lee et al. 2008) with PCR Thermal Cycler DICE (Takara) and GeneAmp PCR system 9700 (Applied Biosystems, CA, USA) (Table 2).

*Statistical analysis*

We performed multiple linear regression analysis between amount of bacterial or virus in river water and seasonality and sampling location by SPSS software (SPSS Ic., Chicago, IL, USA). Briefly, each bacterial concentration and viral titer was transformed in logarithm and considered as response variable, and seasonality (dry or wet season) and sampling location (Metro Manila or Bulacan) as explanatory variables.

*Phylogenic Analysis*

We sequenced PCR product for group A rotavirus (Lee et al. 2008) and hepatitis A virus (Hong et al. 2008) using same PCR primers in Table2. PCR positive samples were purified with SUPREC-PCR kit (TaKaRa, Shiga, Japan) and underwent sequence analysis with BigDye Terminator Cycle Sequence Ready Reaction kit (Applied Biosystems, CA, USA) and 3130 Genetic Analyzer (Applied Biosystems, CA, USA; HITACHI, Tokyo, Japan). Then, we generated phylogenic tree by neighbor joining method with MEGA4.0 software. Reference strains for rotavirus

VP7 partial gene were obtained from Genebank and for hepatitis A virus were from another study in Vietnam (Hong et al. 2008). We analyzed VP7 gene of rotavirus which determines G serotype and VP1 gene of hepatitis A virus to classify genotype.

## Results

*Bacteriological examination*

We performed bacteriological examination with membrane filter method and calculated Colony Forming Unit (CFU)/ml for total coliform for *E.coli*, and *enterococcus*. The concentration of each bacterium in April, August, and December was shown in Figure 3 and 4. The total coliform concentration, except for Marilao, was the highest in April. But for *E. Coli* and *Enterococcus*, there was no apparent seasonality. In terms of geographical distribution, samples collected from Metro Manila (Las piñas and Parañaque) and Meycauayan tend to have higher concentration than samples collected from Marilao and Bocaue, except for in December.

*Multiple linear regression analysis for bacterial concentration*

We performed multiple linear regression analysis for concentration of three bacteria (Table3). We could not confirm statistically significant seasonality difference for the concentrations in our data. However, the concentration in Metro Manila was statistically higher than that of Bulacan in all bacteria ($p<0.05$)

*Viral detection*

We were able to detect adenovirus, enterovirus, hepatitis A virus, norovirus GI, GII, and group A rotavirus by real time PCR, and astrovirus by conventional PCR. Viral titer of each virus by seasonality and sampling locations were shown in Figure 5 to10 except for astrovirus. All viruses had higher titer in dry season (April and December) than wet season (August). For sampling locations, the viral titer in

Metro Manila was higher than Bulacan in all viruses except enterovirus (Figure 6). The titer of norovirus GI was higher than that of norovirus GII.

*Multiple linear regression Analysis for viral titer*

We analyzed viral titer by multiple regression analysis (Table 4). We confirmed that virus had higher titer in dry season than wet season with statistical significant ($p<0.05$). The viral titer in Metro Manila was also statistically higher ($p<0.05$) than Bulacan in all viruses except enterovirus.

*Sequence analysis for group A rotavirus and hepatitis A virus*

For all positive samples by nested PCR for rotavirus and hepatitis A viruses, we performed sequence analysis. All detected rotaviruses belonged to G serotype 1 (Figure11). All detected hepatitis A viruses belonged to genotype IA (Figure12).

## Discussion

We were able to quantify the series of bacteria and viruses in the environmental water in the urban setting of the Philippines. This study was conducted based on our hypothesis that viruses detected from river water reflect the viruses circulating in the population in each area because patients shed viruses into their feces.

As we expected, river water for all sampling sites had high concentration of total coliform, which indicated that water was highly contaminated with feces. The level of total coliform was much higher than the river water and was comparable to sewage water in developed country (Masuda et al. 2003). Although improved sanitation facilities, such as flush pouring to septic tank or piped sewer system and composting toilet, being available for 81% of people living in urban area in the Philippines (WHO 2004), it is important to provide water sanitation with people living along with river, because their garbage directly pollute river. We concluded that sewage treatment system and coverage need improvement.

Although we were able to see the change of the bacterial concentration by different season of the Philippines, we could not reveal apparent seasonality of bacterial concentration by statistical analysis. We expected that the bacterial concentration of dry season would be higher than that of wet season, because of pathogens can be relatively concentrated in dry season. Masuda showed that the bacterial concentration in sewage water become higher when it rained (Masuda et al. 2003). Therefore, we may need to pay attention to the amount of river flow and weather prior to the sampling in order to unify the river condition. On the other side, we showed that concentration of bacteria in Metro Manila was statistically higher than that in Bulacan. This result stated that fecal pollution on river water

depended on population live in the water basin.

As far as we know, there has been no published data of enteric viruses in environmental water in the Philippines. In the current study, we were able to detect adenovirus, enterovirus, hepatitis A virus, norovirus GI, GII, group A rotavirus, and astrovirus, and quantified these viruses except for astrovirus. In developed countries, enteric viruses, such as norovirus, adenovirus, enterovirus, and rotavirus, had been detected from water sample (Lodder et al. 2005; Lee et al. 2008). But developing countries, viral pollution in environmental water has not been well studied.

In comparison of Filipino river water with those in developed countries, the viral concentration in our study was higher. In Italy, the norovirus concentration in sewage samples were $6.80\times10^{2}$ – $6.77\times10^{4}$ copies/ml, and $7.60\times10^{1}$ – $2.40\times10^{3}$ copies/10L in sea water (Giuseppina et al. 2009). Where as in Japan, river water had concentration of $8.3\times10^{1}$ – $1.5\times10^{5}$ copies/100ml (Yamamoto et al. 2005). As mentioned above, the sewage treatment system needs some improvement also from virological perspective.

From our samples, enteric viruses were detected in all season. The titer of adenovirus, hepatitis A virus, norovirus GI, GII, and group A rotavirus were statistically higher in dry season (April and December) than wet season (August). It has shown that rotavirus mainly spread from end of wet season to middle of dry season (October to February) in the Philippines (Urasawa et al. 2008). In our study, rotavirus concentration in December was the highest. This result agreed with the rotavirus study in the Philippines. Our result suggested that the seasonal pattern of the rotavirus in the environmental water was parallel to that of human, but

epidemiological study should be conducted to see the seasonality for rotavirus, as well as other viruses.

Viral titer of adenovirus, hepatitis A virus, norovirus GI, GII, and group A rotavirus were clearly higher in Metro Manila than Bulacan. The population density of Metro Manila is nearly 20 times higher than that of Bulacan province. The result of sampling locations showed that viral titer would depend on the population of each area which in lines with our data on fecal pollution. But in this study, only enterovirus had pattern from other viruses. It may be due to the fact that the primer pair used for enterovirus detection can detect enteroviruses in animals such as Swine Vesicular Disease Virus (SVDV) based on the homology of the primer, therefore titer of enterovirus could be affected by these non-human pathogenic viruses. Bulacan region has more farm than Metro Manila. The compost from farm could contaminate to river water. We may need to subtype enterovirus.

Generally, specific type of norovirus (GII/4) is mainly detected from clinical samples in outbreak all over the world (Roger et al. 2009; Cheryl et al. 2009). But both GI and GII norovirus have been detected from environmental water (Saitoh et al. 2007; Yamamoto et al. 2005). We also detected both norovirus GI and GII from river water in the Philippines. It may suggest that various types of norovirus certainly exist in the community. It is important to research environmental sample to detect viruses which could not be detect from clinical samples.

We performed sequence analysis for group A rotavirus VP7 gene and hepatitis A virus VP1 gene which were able to be detected by nested PCR. Detected rotavirus belonged to G serotype 1. G1 to G4 rotavirus was usually detected from all over the world, so this G1 rotavirus spread in the Philippines too. The study that research

rotaviruses spreading in the Philippines in 1990s, showed G1 to G4 rotavirus is mainly spread in the community in 1995-1998 season (Urasawa et al. 2008). According to that study, different serotype of group A rotavirus can circulate by year. But in this study, only one serotype was detected in March, April and December samples, and we could not see serotype shift in our study. For hepatitis A virus, all viruses belonged to genotype IA which is the most major genotype all over the world. It is believed that these viruses detected from river water samples reflect viruses circulating in population. Therefore we showed that rotavirus and hepatitis A virus spreading in the Philippines were same type to the virus circulating among human all over the world.

From our study in the Philippines, many enteric viruses were detected from urban river water, which indicate that there is risk of viral infection from environmental water. It is important that government provide information of risk of water pollution and effort to improve water quality, to improve public health condition. Since there is a cycle of pathogens between human and environment, we also should consider the prevalence of viruses among human population in the study areas. But we could not access circulating viruses in human population, because we do not have clinical samples collected from diarrheal cases. We need to study environmental and clinical sample at same time to obtain more information about ecological cycle of these viruses.

## Conclusion

From this study, we showed high titer of enteric viruses in urban river water of the Philippines and there is the risk that waterborne infection from environmental water. The result from analysis of environmental water showed seasonal and sampling locations of viral titer and existence of viruses circulating among population silently. Therefore, we need to survey viruses in environmental water and viruses circulating in human population at same period.

## Acknowlegments

I would like to thank Tohoku university staffs in the Philippines and staffs of Research Institute for Tropical Medicine (RITM, the Philippines) for supporting my water sampling and experiment in the Philippines. I am indebted to prof. Mitsuo Kaku for reviewing my master thesis.

## Reference


Celia C. Carlos, Mediadora C. Saniel (1990) Etiology and Epidemiology of Diarrhea Phil J Microbiol Infect Dis, 19(2), 51-53

Cheryl S. Y. Li, Paul K. S. Chan, Julian W. Tang (2009) Prevalence of Eiarrhea Viruses in Hospitalized Children in Hong Kong J Medical Virology, 81, 1903-1911

Cunliffe NA., Nakagomi O. (2005) A critical time for rotavirus vaccines: a review. Expert Rev Vaccines 4(4), 521-532

Franca Baggi, Raffaele Peduzzi (2000) Genotyping of Rotaviruses in Environmental Water and Stool Samples in Southern Switzerland by Nucleotide Sequence Analysis of 189 Base Pairs at the 5' End of the VP7 Gene. J Clinical Microbiology, Vol.38, No.10, 3681-3685

Heim A, Ebnet C, Harste G, Pring-Akerblom P. (2003) Rapid and quantitative detection of human adenovirus DNA by real-time PCR. J Med Virol 70, 228–239.

Hsiao-Chuan Lin, Chuan-Liang Kao, Luan-Yin Chang, Yu-Chia Hsieh, Pei-Lan Shao, Ping-Ing Lee, Chun-Yi Lu, Chin-Yun Lee, Li-Min Huang (2008) Astrovirus Gastroenteritis in Children in Taipei. J Formos Med Assoc, Vol.107, No.4, 295-303

Japan Sewage Works Association (1997), How to test sewagewater

Jin Xu, Yi Yang, Jiae Sun, Yunzhen Ding (2009) Molecular Epidemiology of Norovirus Infection Among Children With Acute Gastroenteritis in Shanghai, China, 2001-2.005. Journal of Medical Virology, 81, 1826-1830

Kaori Nakanishi, Takeshi Tsugawa, Shinjiro Honma, Shuji Nakata, Masatoshi Tatsumi, Yuko Yoto, Hiroyuki Tsutsumi (2009) Detection of enteric viruses in rectal swabs from children with acute gastroenteritis attending the pediatric outpatient clinics in Sapporo, Japan Journal of Clinical Virology, 46, 94-97

Kazuyoshi Yano (2006) Pollution of Water and food by Enteric Virus. Journal of Water EnvironmentVol.29 No.3 124-129

Kultz J., Lee T. (1978) Astrovirus gastroenteritis age distribution of antibody. Med Microbiol Immunol, 166, 227-230

Lee, Cheonghoon and Sang-Jong Kim. 2008. Molecular Detection of Human Enteric Viruses in Urban Rivers in Korea. J. Microbiol. Biotechnol. 18(6), 1156-1163.

Lodder W. J., de Roda Husman, A. M. (2005) Presence of norovirus and other enteric viruses in sewage and surface waters in the Netherlands. Applied and Environmental Microbiology, 71 (3), 1453-1461

Marilla G. Lucero, Mediadora C. Saniel, Josefina G. Geronimo, Corgazon V. Ang, Fe T.

Leano, Rosa B. Mate, Elizabeth C. Trajano, Ma Elinor G. Sanvictores, Zenaida J. Forbes and Thelma E. Tupasi (1984) Etiology of Diarrhea in Hospitalized Children. Phl J Microbiol Infect Dis 1984, 13(1), 17-24

Mika Saitoh, Hirokazu Kimura, Kunihisa Kozawa, Osamu Nishio, Akira Shoji (2007) Detection and Phylogenetic Analysis of Norovirus in *Corbicula fluminea* in a Freshwater River in Japan. Microbio. Immunol., 51(9), 815-822

Mineyuki Okada, Tomoko Ogawa, Ikuo Kaiho, Kuniko Shinozaki (2005) Genetic Analysis of Noroviruses in Chiba Prefecture, Japan, between 1999 and 2004. Journal of Clinical Microbiology, Vol.43, No.9, 4391-4401

Ministry of Health, Labour and Welfare (2007) Method of Norovirus Detection. http://www.mhlw.go.jp/topics/syokuchu/kanren/kanshi/dl/031105-1a.pdf

Monpoeho, S., Dehee, A., Mignotte, B., Schwartzbrod, L., Marechal, V., Nicolas, J.C., Billaudel, S., Ferre, V., (2000) Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR. Biotechniques 29, 88–93.

National Astronomical Observatory of Japan(2006) Chronological Scientific Tables, 2007

Osamu Nishio, Miho Akiyama, Yumiko Kato (2002) Detection of Hepatitis A virus by Real time PCR. Summaries of The 76th Annual Meeting of the Japanese Association of Infectious Disease2002 ; 251

Philippine Atmospheric, Geophysical and Astronomical Services Administration Monthly precipitation data in Quezon city, 2009

Phuc Le Hoang , Khanh Huu Trong , Tung Thanh Tran , Tran Thien Tuan Huy and Kenji Abe. (2008) Detection of hepatitis A virus RNA from children patients with acute and fulminant hepatitis of unknown etiology in Vietnam: Genomic characterization of Vietnamese HAV strain. Pediatrics International 50, 624-627.

Roger I. Glass, Umesh D. Parashar, and Mary K. Estes. (2009) Norovirus Gastroenteritis. The New England Journal of Medicine. 361:1776-85.

Sumio Masuda,Masahito Yamauchi,Sota Watanabe,Nobuhiro Kurayama, Yutaka Dote ,Toshiro Maruyama (2003)Research of Pollution by *E. Coli* in Urban Sewage Water, Papers of Environmental Engineering Vol.40

Susumu Kato (1999) Quality of Environmental Water in the Philippines, Report of Environmental Science facility in Mie prefecture Vol.9

Tiong Gim Aw, Karina Yew-Hoong Gin, Lynette Lin Ean Oon, Eileen Xueqin Chen, Chee Hoe Woo (2009) Prevalence and Genotypes of Human Noroviruses in Tropical Urban Surface Waters and Clinical Samples in Singapore. Applied and Environmental Microbiology, Vol.75, No.15, 4984-4992

Toshio Yamamoto, Tomohiro Shimazu, Yoshihiro Endo, Hiroyuki Shiraishi, Tsutomu Kageyama (2005) Quantification of Norovirus in Rivers inflowing to oyster culture water in Miyagi. Journal of Water EnvironmentVol.28, No.8, 515-521

Tsutomu Kageyama, Shigeyuki Kojima, Michiyo Shinohara, Kazue Uchida, Shuetsu Fukushi, Fuminori B. Hoshino, Naokazu Takeda, and Kazuhiko Katayama. (2003) Broadly Reactive and Highly Sensitive Assay for Norwalk-Like Viruses Based on Real-Time Quantitative Reverse Transcription-PCR. Journal of Clinical Microbiology vol.41 No.4 p.1548-1557.

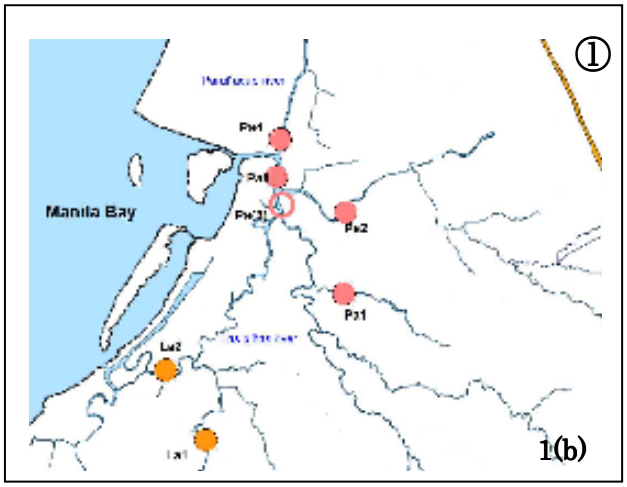
Tuan Anh Nguyen, Fumihiro Yagyu, Michio Okame, Tung Gia Phan, Quang Duy Trinh, Hainian Yan, Kim Trong Hoang, Anh Thi Huynh Cao, Phuc Le Hoang, Shoko Okitsu, Hiroshi Ushijima (2007) Diversity of Viruses Associated With Acute Gastroenteritis in Children Hospitalized with Diarrhea in Ho Chi Minh City, Vietnam. Journal of Medical Virology, 79, 582-590

Tung Gia Phan, Michio Okame, Tuan Anh Nguyen, Niwat Maneekarn, Osamu Nishio, Shoko Okitsu, Hiroshi Ushijima (2004) Human Astrovirus, Norovirus (GI, GII), and Sapovirus Infections in Pakistani Children With Diarrhea. Journal of Medical Virology, 73, 256-261

Urasawa S., Celia Carlos, Taniguchi K., Rose Mate, Kobayashi N., Urasawa T. (2008) Clinico-epidemiological Study of Rotavirus Diarrhea in Wuhan, China and Muntinlupa, Philippines –Part II Study in Muntinlupa, Philippines- Clinical Pediatric MedicineVol.56 (3,4)63-76

Xiaoli L. Pang,1 Bonita Lee,1 Nasim Boroumand,1 Barbara Leblanc,1 Jutta K. Preiksaitis,1 and Charlotte C. Yu Ip. (2004) Increased Detection of Rotavirus Using a Real Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assay in Stool Specimens From Children With Diarrhea. Journal of Medical Virology 72:496–501.

WHO (2004) Country, regional and Global Estimates on Water and Sanitation

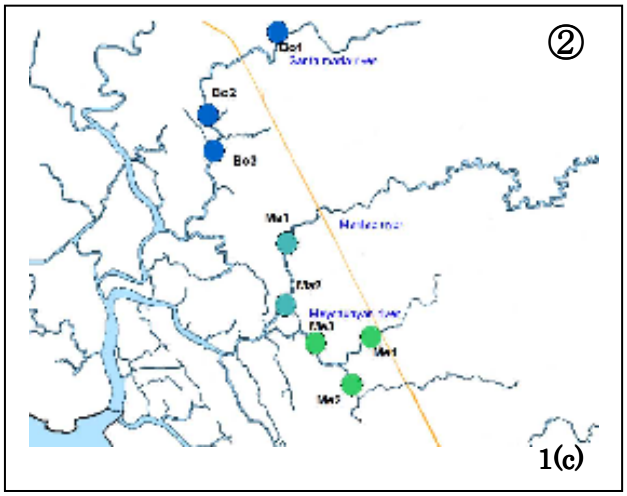




Figure1: Sampling sites.

1(a) shows map of Luzon, 1(b) shows map of Metro Manila sampling area, and 1(c) shows map of Bulacan sampling area.

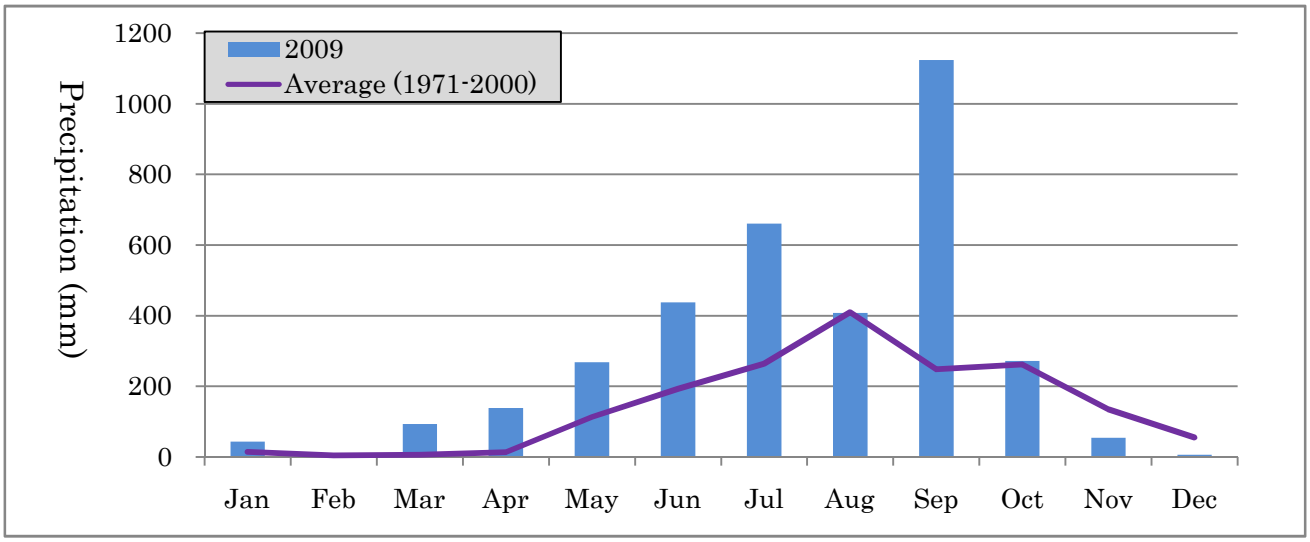



Figure2: The average of monthly precipitation in the Philippines between 1971 and 2000, and 2009.

| Virus | Sequence (5' → 3') | Reference |
|---|---|---|
| Adenovirus | | |
| AdV F | GCCACGGTGGGGTTTCTAAACTT | Heim et al., 2003 |
| AdV R | GCCCCAGTGGTCTTACATGCACATC | |
| AdV Probe | FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-TAMRA | |
| Enterovirus | | |
| EV F | GATTGTCACCATAAGCAGC | Monpoeho et al., 2000 |
| EV R | CCCCTGAATGCGGCTAATC | |
| EV Probe | FAM-CGGAACCGACTACTTTGGGTGTCCGT-TAMRA | |
| Norovirus GI | | |
| NV1 F | CGYTGGATGCGNTTYCATGA | Kageyama et al., 2003 |
| NV1 R | CTTAGACGCCATCATCATTYAC | |
| NV1 Probe(a) | FAM-AGATYGCGATCYCCTGTCCA-TAMRA | |
| NV1 Probe(b) | FAM-AGATCGCGGTCTCCTGTCCA-TAMRA | |
| Norovirus GII | | |
| NV2 F | CARGARBCNATGTTYAGRTGGATGAG | Kageyama et al., 2003 |
| NV2 R | TCGACGCCATCTTCATTCACA | |
| NV2 Probe | FAM-TGGGAGGGCGATCGCAATCT-TAMRA | |
| Group A Rotavirus | | |
| RV F | ACCATCTACACATGACCCTC | Pang et al., 2004 |
| RV R | GGTCACATAACGCCCC | |
| RV Probe | FAM-ATGAGCACAATAGTTAAAAGCTAACACTGTCAA-TAMRA | |
| Hepatitis A virus | | |
| HAV F | AGGGTAACAGCGGCGGATAT | Nishio et al., 2002 |
| HAV R | ACAGCCCTGACARTCAATYCACT | |
| HAV Probe | FAM-AGACAAAAACCATTCAACRCCGRAGGAC-TAMRA | |

Table1: Primer and Probe for real time PCR.

| Virus | Sequence (5' → 3') | Reference |
|---|---|---|
| Group A Rotavirus | | |
| R1 | GGCTTTAAAAGAGAGAATTTCCGTCTGG | Cheonghoon et al. 2008 |
| END9 | GGTCACATCATACAATTCTAATCTAAG | |
| R3* | GTATGGTATTGAATATACCAC | |
| Rp* | TCCATTGATCCTGTTATTGG | |
| Hepatitis A virus | | |
| VP1 OF | ATTCAGATTAGACTGCCTTGGTA | Hong et al. 2008 |
| VP1 OR | CCAAGAAACCTTCATTATTTCATG | |
| VP1 IF* | GCAAATTACAATCATTCTGATGA | |
| VP1 IR* | CTTCYTGAGCATACTTKARTCTTTG | |
| Astrovirus | | |
| Ast1 | CCTGCCCCGAGAACAACCAAG | Cheonghoon et al. 2008 |
| Ast2 | GTAAGATTCCCAGATTGGT | |
| Ast3-A1* | CCTTGCCCCGAGCCAGAA | |
| Ast4-A2* | TATTCACAAACTTATGGCAA | |

Table2: Primer pairs for conventional PCR.

*Primer pair used for nested PCR.

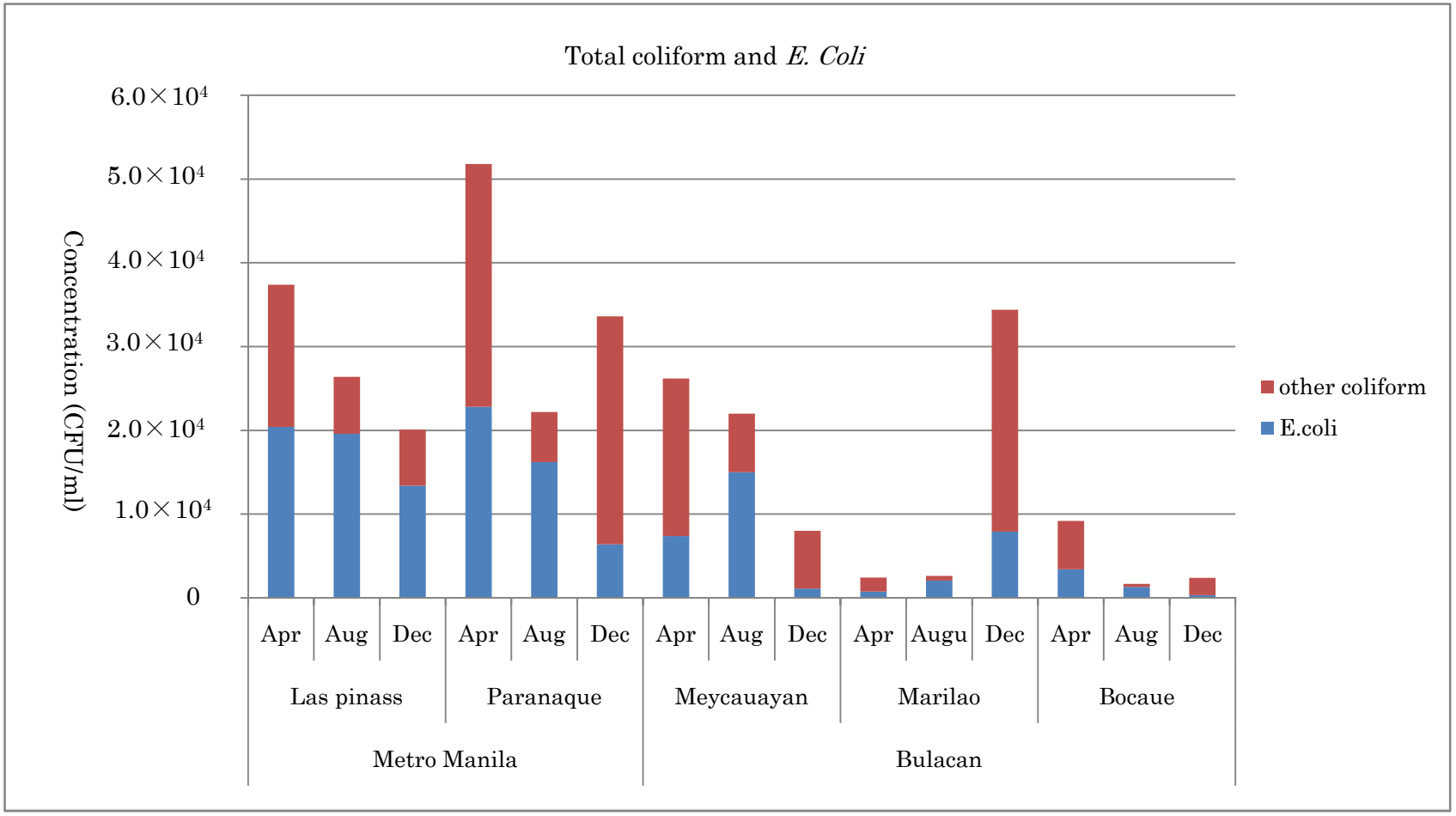



Figure3: Concentrations of total coliform and *E. Coli* in Metro Manila and Bulacan in each season.

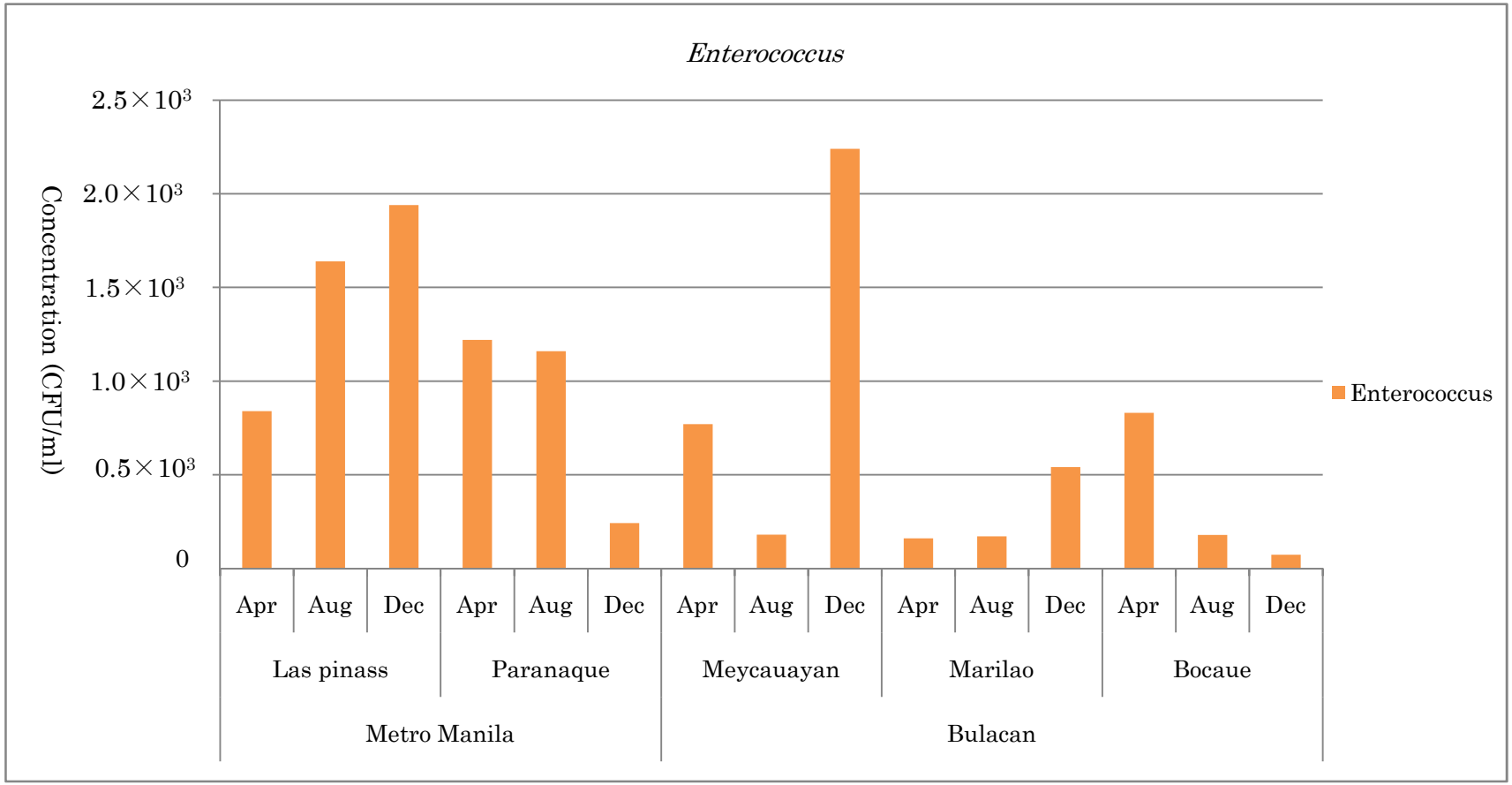



Figure4: Concentration of *Enterococcus* in Metro Manila and Bulacan in each season.

| | Model | Unstandardized coefficients | | Significant |
|---|---|---|---|---|
| | | B | Standard Error | |
| Total coliform | Metro Manila | 0.693 | 0.139 | <0.001 |
| | Dry season | 0.235 | 0.141 | 0.107 |
| | Constants | 3.632 | 0.126 | <0.001 |
| *E.Coli* | Metro Manila | 0.855 | 0.151 | <0.001 |
| | Dry season | -0.177 | 0.153 | 0.258 |
| | Constants | 3.437 | 0.137 | <0.001 |
| *Enterococcus* | Metro Manila | 0.549 | 0.13 | <0.001 |
| | Dry season | 0.143 | 0.129 | 0.274 |
| | Constants | 2.382 | 0.111 | <0.001 |

Table3: The result of multiple linear regression analysis about indicator bacteria.

B means efficacy of seasonality or sampling locations, B>0 means that the factor positively affects to bacterial concentration and B<0 means negative effect. Larger number means more effective to bacterial concentration.

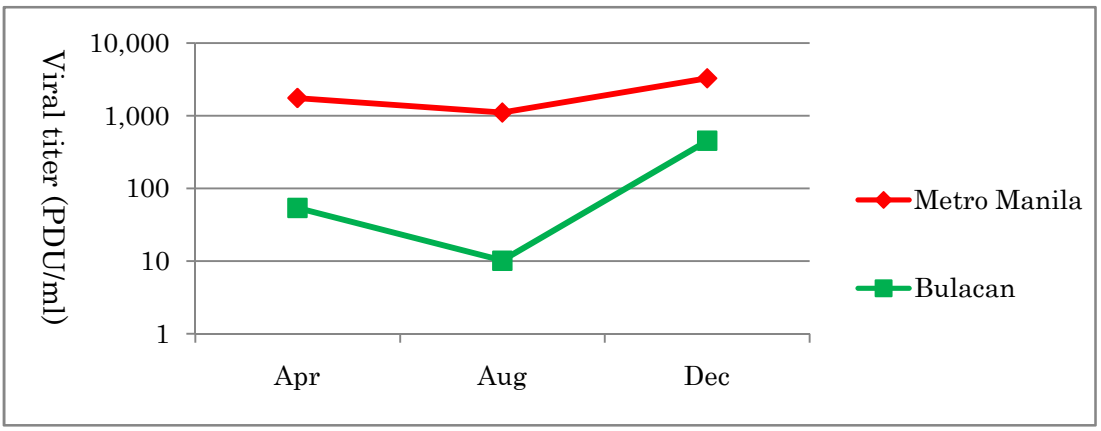



Figure5: Adenovirus titer by seasonality and sampling locations.

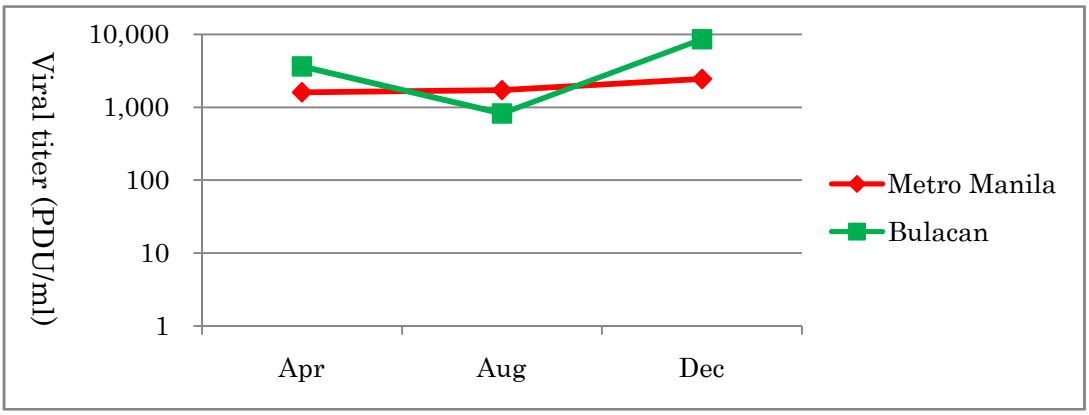



Figure6: Enterovirus titer by seasonality and sampling locations.

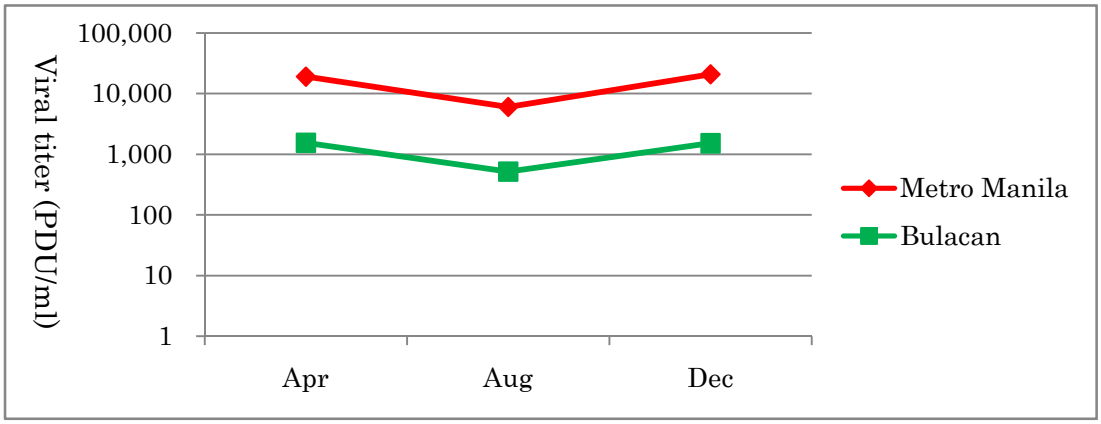



Figure7: Hepatitis A virus titer by seasonality and sampling locations.

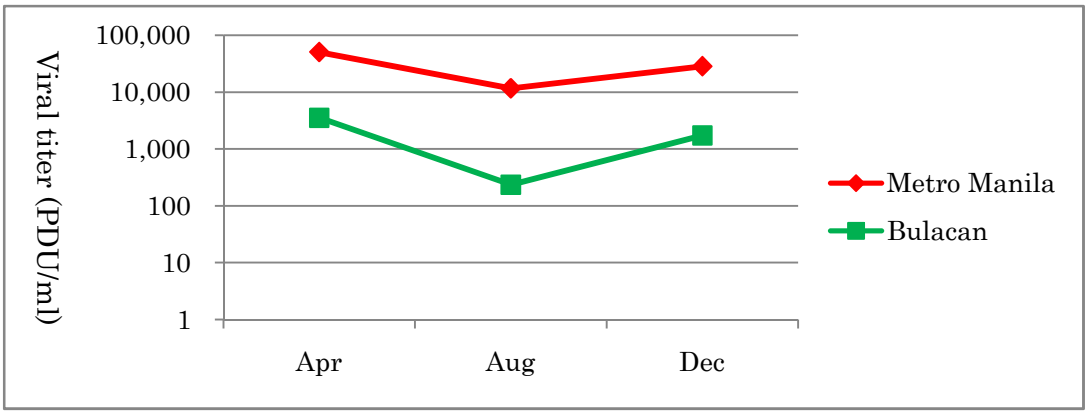



Figure8: Norovirus GI titer by seasonality and sampling locations.

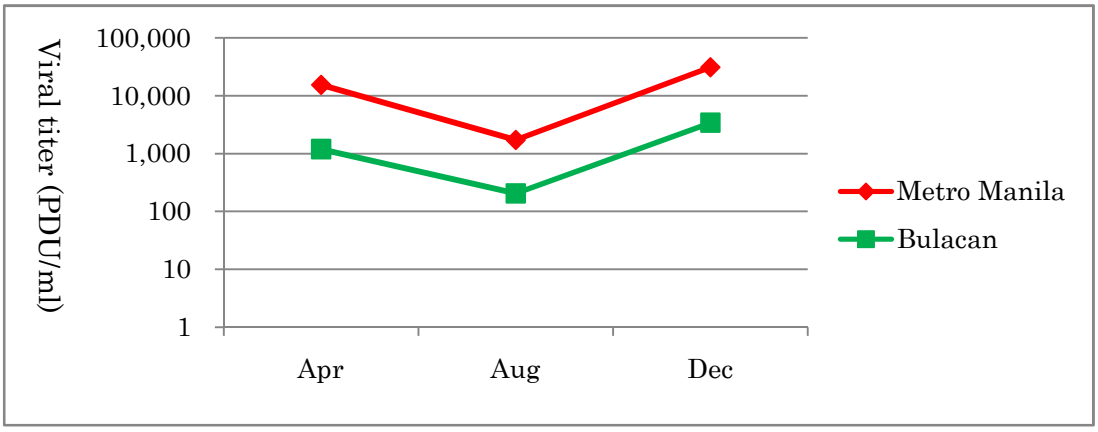



Figure9: Norovirus GII titer by seasonality and sampling locations.

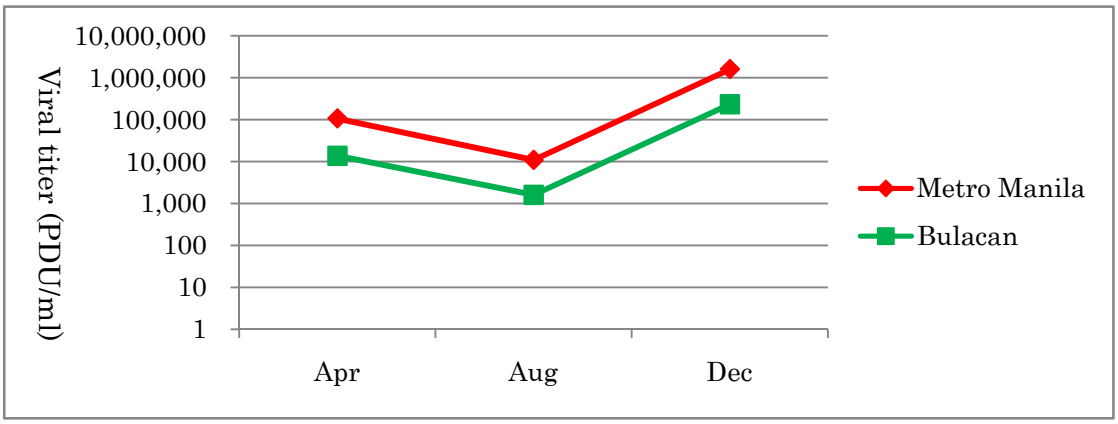



Figure10: Group A rotavirus titer by seasonality and sampling locations.

| | Model | Unstandardized coefficients | | Significant |
|---|---|---|---|---|
| | | B | Standard Error | |
| Adenovirus | Metro Manila | 1.210 | 0.144 | <0.001 |
| | Dry season | 0.324 | 0.149 | 0.033 |
| | Constants | 1.700 | 0.171 | <0.001 |
| Enterovirus | Metro Manila | 0.005 | 0.091 | 0.954 |
| | Dry season | 0.448 | 0.096 | <0.001 |
| | Constants | 2.886 | 0.087 | <0.001 |
| Hepatitis A virus | Metro Manila | 0.709 | 0.104 | <0.001 |
| | Dry season | 0.477 | 0.110 | <0.001 |
| | Constants | 2.929 | 0.114 | <0.001 |
| Norovirus GGI | Metro Manila | 1.074 | 0.073 | <0.001 |
| | Dry season | 0.465 | 0.085 | <0.001 |
| | Constants | 2.930 | 0.092 | <0.001 |
| Norovirus GGII | Metro Manila | 0.910 | 0.076 | <0.001 |
| | Dry season | 0.902 | 0.088 | <0.001 |
| | Constants | 2.376 | 0.091 | <0.001 |
| Group A rotavirus | Metro Manila | 0.815 | 0.172 | <0.001 |
| | Dry season | 1.431 | 0.196 | <0.001 |
| | Constants | 3.050 | 0.199 | <0.001 |

Table4: The result of multiple linear regression analysis about enteric viruses.

B means efficacy of seasonality or sampling locations, B>0 means that the factor positively affects to viral titer and B<0 means negative effect. Larger number means more effective to viral titer.

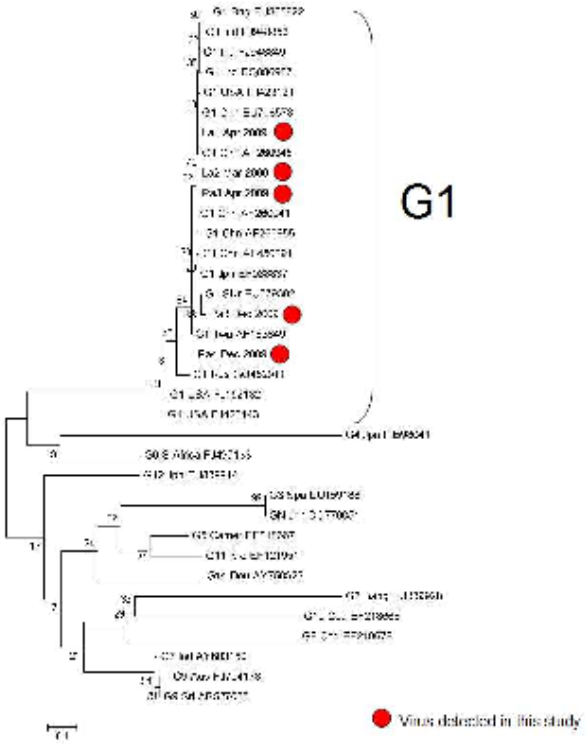



Figure11: Phylogenic tree about group A rotavirus VP7 gene (147bp).

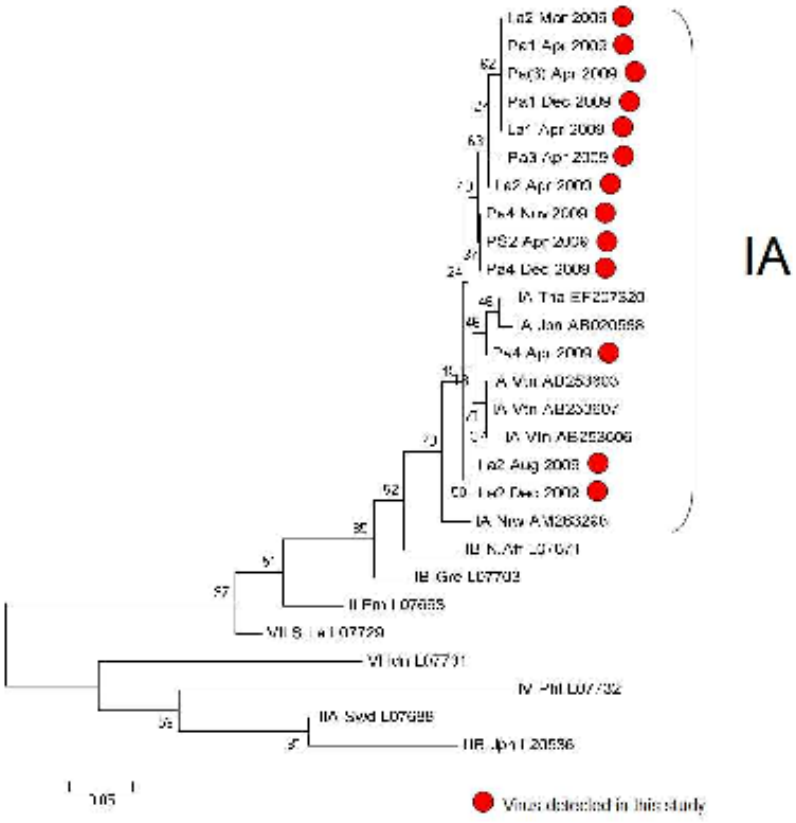



Figure12: Phylogenic tree about hepatitis A virus VP1 gene (107bp).