Detection of enteric viruses in five important tributaries of Laguna Lake, Philippines by PCR assays

Joy Ann P. Santos[1,2], Maribell A. Dollete[3], Nadine Janelle E. Sinco[2], Ma. Jowina H. Galarion[4], Windell L. Rivera*[,1]

[1]Pathogen-Host-Environment Interactions Research Laboratory, Institute of Biology, College of Science, University of the Philippines Diliman, Quezon City 1101, Philippines
[2]Biological Research and Services Laboratory, Natural Sciences Research Institute, University of the Philippines Diliman, Quezon City 1101, Philippines
[3]Manila HealthTek, Inc., Marikina City 1800, Philippines
[4]Institute of Molecular Biology and Biotechnology, National Institutes of Health, University of the Philippines Manila, City of Manila 1000, Philippines

ABSTRACT

Fecal contamination of water sources caused by humans and animals presents public health risks due to the presence of pathogens. Enteric viruses are important waterborne pathogens since their occurrence in contaminated water is subsequently linked to outbreaks. However, detection of these viruses is not routinely done in water quality analyses, and there is a paucity of reports on the surveillance of these pathogens. Hence establishing a baseline data for these viruses is of utmost importance. Water samples from five important tributaries of Laguna Lake in the Philippines were collected to detect the presence of human adenoviruses, noroviruses, hepatitis E and A, and rotaviruses. Samples were processed via double filtration method using 0.45 µm and 0.2 µm nitrocellulose membrane filters. Viral DNA were extracted from the 0.22 µm membranes using commercially available DNA extraction kit before doing polymerase chain reaction (PCR) assays. Real-time PCR assays showed that human adenoviruses, noroviruses, *Hepatitis E*, rotaviruses, astroviruses, and enteroviruses were found in the tributaries. Phylogenetic analyses of the identified adenoviruses and astroviruses revealed that they were of human origin indicating human sources of contamination in the lake. A simple filtration method coupled with a real-time PCR assay can be used to detect common enteric viruses in water samples. Identifying the sources of fecal contamination in bodies of water will contribute to an efficient risk assessment of environmental systems.

*Corresponding author
Email Address: wlrivera@science.upd.edu.ph
Date received: 13 January 2021
Date revised: 10 May 2021
Date accepted: 20 May 2021

KEYWORDS

enteric viruses, detection, Laguna Lake, Philippines, real-time PCR

INTRODUCTION

Waterborne diseases, caused by bacteria, viruses, and protozoa, have been the major cause of many outbreaks in different parts of the world (Craun et al. 2006). Human enteric viruses can reproduce only through a human host and can be transmitted through fecal-oral route (Cliver 1997). One of the most significant characteristics of enteric viruses is that they can be transmitted from person to person at a low infectious dose (<20

particles), enough to cause illness (Warriner 2005). Commonly studied groups of enteric viruses belong to the families *Picornaviridae* (polioviruses, enteroviruses, coxsakieviruses, *Hepatitis A*, and echoviruses), *Adenoviridae* (adenoviruses), *Caliciviridae* (noroviruses, caliciviruses, astroviruses, and small round-structured viruses), and *Reoviridae* (reoviruses and rotaviruses). Enteric virus groups that are considered to be emerging waterborne pathogens, based on their cellular and molecular structures that make them resistant to current water treatment processes, include circoviruses (consisting of torque *Tenovirus* and torque *Tenovirus*-like virus; these are nonenveloped viruses with single-stranded circular DNA and are resistant to heat inactivation), *Picobirnaviridae* (small nonenveloped viruses with bisegmented double-stranded RNA that are extremely resistant to UV light inactivation), parvoviruses (the smallest known enteric viruses, with single stranded RNA and high heat resistance), and polyomaviruses (including JC virus, BK virus, and simian virus 40; these are nonenveloped double-stranded DNA viruses that have been found to be very heat stable but are less resistant to chlorination than enteroviruses) (Urlings et al. 1993; Wilhelmi et al. 2003).

Immunohistochemical detection, immunofluorescence assay, cell culture, and neutralization testing are conventional approaches for the detection of enteric viruses. These methods are restricted by the number of pathogens they can detect. The absence of antibodies against viral surface proteins leads to many undetected clinical samples. Also, cell culture and neutralization tests are laborious and time-consuming (Piao et al. 2012). Real-time polymerase chain reaction (real-time PCR) is a method by which the amount of PCR product can be determined in real-time via fluorescent dyes (Maddocks and Jenkins 2017). The real-time PCR machine measures the intensity of a fluorescent signal generated by an intercalating dye or from the breakdown of a dye-labeled probe during the amplification of a target sequence. It basically detects the presence or absence of a target nucleic acid as well as measures the concentration of that target in a sample (Stephenson 2016). Real-time PCR can be used to detect and classify all potential infectious pathogens in a given sample. Moreover, real-time PCR assays are used to diagnose enteric virus infections with good sensitivity and specificity (Chen et al. 2006; Thao et al. 2010). Real-time PCR assays, including multiplex assays, have also been performed with promising specificity and sensitivity at a shorter amount of time to detect *Astrovirus* (Zhang et al. 2006; Logan et al. 2007), *Adenovirus* (Bennett and Gunson 2017), *Norovirus* (Logan et al. 2007; Butot et al. 2010; Dung et al. 2013; Neesanant et al. 2013; Yan et al. 2013; Farkas et al. 2015), and *Rotavirus* (Schwarz et al. 2002; Jothikumar et al. 2009; Kottaridi et al. 2012; Dung et al. 2013; Mijatovic-Rustempasic et al. 2016).

Laguna Lake is among the most important inland bodies of water in the Philippines. It is located southeast of Manila and situated along the provinces of Rizal and Laguna. It covers approximately 900 square kilometers of total surface area and 3,820 square kilometers of watershed area. With an approximate retention time of eight months, the primary water inflow of Laguna Lake is derived from catchments and 21 major river tributaries. The lake has 35 shoreland municipalities with a total population of 15 million and is currently being economically used as a water source of business establishments for fisheries production and aquaculture, electricity generation, agriculture, industrial cooling, recreation, and domestic water supply (Department of Environment and Natural Resources – Environment Management Bureau 2014). Due to Laguna Lake's multiple uses, the Laguna Lake Development Authority (LLDA) has implemented water quality monitoring programs since the 1970s to aid in its conservation and management, but rapid urbanization, population growth, and industrialization continue to contribute to the dwindling of its quality. Among the most common sources of pollutants causing the lake's degradation are oil and chemical spills, illegal dumping of wastes, industrial wastewater (e.g. cadmium, lead, mercury, and cyanide), agricultural wastewater (e.g. livestock manure, fertilizers), and domestic sewages (e.g. pathogenic bacteria and viruses, fecal coliforms) (Santos-Borja and Nepomuceno 2005). Hence this present study used both real-time and conventional PCR assays to detect enteric viruses in five important tributaries of Laguna Lake.

MATERIALS AND METHODS

Water collection and nucleic acid extraction

Two liters of each water sample from five tributaries in Laguna Lake were collected at 10 cm below the water surface in sterile containers and transported on ice to the laboratory within 4 h. For nucleic acid extraction, 300 to 500 ml of water samples were filtered using 0.45 µm mixed cellulose membranes and subsequently filtered using 0.2 µm membrane filters (GSWP29325, Merck Millipore, USA) before adding DNA/RNA Stabilization Reagent (Zymo Research). Nucleic acid extractions were done using ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research, USA), following the manufacturer's protocol. For RNA samples, cDNA synthesis was done using SensiFAST cDNA synthesis kit (Bioline, UK) following the manufacturer's protocol.

Real-time and conventional PCR assays

Detection of enteric viruses was done by real-time PCR assays using PowerUP™ SYBR® Green Master Mix (Thermo Fisher Scientific, USA) and CFX96 Touch Real-Time Detection System (BioRad, USA). Conventional PCR assays were also done in parallel using Gotaq® Green Mastermix (Promega, USA) in SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific). Oligonucleotide primers used in this study are listed in Table 1.

Sequencing and phylogenetic analysis

DNA sequencing was performed using ABI PRISM BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Fister City, CA) on an automated sequencer (ABI PRISM 3100 model; Applied Biosystems).

Reference sequences were gathered for the different genera of *Adenoviridae* (*Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus,* and *Siadenovirus*) and *Astroviridae* (*Avastrovirus* and *Mamastrovirus*) from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). Sequences were aligned in BioEdit using the Clustal W program. The aligned sequences were trimmed as needed and used to test for the most appropriate model of DNA substitution using jModelTest. TIM2+Γ was selected as the final model using the Akaike Information Criterion. The Neighbor-Joining tree was created using PAUP* (Swofford 2003). The following parameters were used: bootstrap replicate = 1000, alpha = 0.671. PAUP* was also used to create the Maximum Parsimony tree. The following parameter was used: bootstrap replicate = 1000. Maximum Likelihood trees were constructed using PhyML v.3.0 (Guindon et al. 2010). Bootstrap replicates of 1000 were used to calculate probability values. Meanwhile, Bayesian inference trees were constructed using MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003) using these parameters: 1M generations, blockchain temperature of 0.2000. The trees were visualized using TreeExplorer v.2.12 (Tamura 1999).

Table 1: Oligonucleotide primers used for detection of enteric viruses by conventional PCR and real-time PCR assays.

	Primer name	Target gene	Sequence	Assay	Reference
Human adenoviruses	Forward	*hexon*	TACATGCAYATCGCCG	Real-time PCR	Prevost et al. 2015
	Reverse		CGGGCRAAYTGCACC		
	Hex A	*hexon*	GCC GCA GTG GTC TTA CAT GCA CAT C	Conventional PCR	Biscaro et al. 2018
	Hex B		CAG CAC GCC GCG GAT GTC AAA GT		
Noroviruses	NVGI_F5290	*orf1–orf2 junction*	CGYTGGATGCGSTTCCAT	Real-time PCR	Prevost et al. 2015
	NVGI_R5374		CTTAGACGCCATCATCATTTAC		
	HEV_R		AGGGGTTGGTTGGATGAA		
Rotavirus	RAV_F9	*nsp3*	ATGSTTTTCAGTGGTTGMTGC	Real-time PCR	Prevost et al. 2015
	RAV_R83		AGCDACAACTGCRGCTTC		
Hepatitis E	HEV_F	*capsid*	GGTGGTTTCTGGGGTGAC	Real-time PCR	Jothikumar et al. 2009
	HEV_R		AGGGGTTGGTTGGATGAA		
Astroviruses	Mon269	*orf2*	CAA CTC AGG AAA CAG GGT GT	Conventional PCR	Biscaro et al. 2018
	Mon270		TCA GAT GCA TTG TCA TTG GT		
Enteroviruses	F1		CAA GCA CTT CTG TTT CCC CGG	Conventional PCR	Biscaro et al. 2018
	R1		ATT GTC ACC ATA AGC AGC CA		

RESULTS AND DISCUSSION

Laguna Lake is the largest inland water resource in the Philippines. It is a source of potable water supply for municipalities such as Alabang, Bayanan, Poblacion, Putatan, Tunasan, and New Alabang. The lake is also used in irrigation for agriculture, water source for industrial cooling, power generation, transport route, floodwater reservoir as well as a location for tourism and recreation. The lake has a major role in aquaculture as a source of fish, molluscs, and crustaceans and serves as a biological source of food for humans and feeds for other animals (Bocci 1999; Bajet et al. 2012; Atienza et al. 2015; Water Technology 2019). Due to public health and safety threats that they pose, sources of contamination within the lake must be studied.

Five important tributaries were chosen for this study: Alabang-Cupang River, Bayanan Creek, Tunasan River, San Isidro River, and Biñan River. The water inflow of Laguna Lake is partly derived from these river tributaries, possibly contaminating the lake. Figure 1 shows the map with the sampling sites and corresponding viruses detected in each site. Real-time PCR assays showed the presence of human adenoviruses (HAdVs) in two of the sites, Alabang-Cupang River and Biñan River, while three sites, Alabang-Cupang River, Bayanan Creek, and Biñan River, were positive for noroviruses. *Hepatitis E* was present in three sites, Alabang-Cupang River, Bayanan Creek, and San Isidro River. All five sites were positive for rotaviruses (Figures 2A and 2B). Conventional PCR assays showed positive results for astroviruses (Figure 2C) and enteroviruses for Alabang-Cupang River and Bayanan Creek. The Alabang-Cupang River had the greatest number of enteric viruses identified. Sequence analysis further validated the presence of HAdVs in sites 1 and 5 and astroviruses in sites 1 and 2. In addition, molecular phylogenetic analysis showed that the HAdVs detected belong to type 41, while the astroviruses isolates claded with human astroviruses (Figure 3). Thus the presence of these enteric viruses in Laguna Lake strongly indicates human sewage contamination.

Waterborne enteric viruses are a growing concern to public health due to their tolerance to wastewater treatment processes. Human adenovirus is the major indicator of fecal contamination in water and the most common etiologic agent for gastroenteritis in children (Dona et al. 2016; Rames et al. 2016). Also, rotaviruses pose a risk on the health of newborns and immunosuppressed individuals (Pereira et al. 2013; Prevost et al. 2015). Moreover, viruses such as human adenoviruses, noroviruses, hepatitis E and A, and rotaviruses have been previously linked to viral-associated outbreaks of waterborne diseases (Prevost et al. 2015). This is probably due to the stability of viruses in water as well as their higher infectivity rates compared to bacteria. Thus enteric viruses should be included in water quality analyses.

Analysis of waterborne enteric viruses requires water sample collection, water concentration, virus detection, and identification. However, the low quantity of these viruses in a large volume of water has been a technical challenge. Thus, processing of water concentration is important. To detect viruses, one of the methods is to identify the presence of viral nucleic acid using molecular amplification techniques such as conventional PCR or real-time PCR. A wide range of analytical methods is available for virus detection in water samples, and most of the data collected are obtained by PCR (Bosch et al. 2011). Conventional PCR can be useful for several samples since it produces several amplicons and is less expensive than other methods. By contrast, real-time PCR is highly specific and adaptable and provides faster turnaround time.

PCR products from the *orf2* gene of two *Astrovirus* isolates from two sites revealed that these were from human origin (Figure 3A). Meanwhile, analysis of the *hexon* gene of the two HAdV isolates from two sites revealed that both isolates belonged to the human adenovirus species F (HAdV-F) type 40 (Figure 3B). Together with type 41, these HAdV-F are commonly associated with acute diarrheal disease (ADD). ADD has a major impact on global health due to the high cost of hospitalizations and outpatient visits associated with diarrhea. Even in developed countries, ADD is still associated with high morbidity, mostly among children as well as the elderly, while it is the second leading cause of morbidity and mortality in children under 5 years of age in developing countries (Reis et al. 2016).

Using PCR, this study reports for the first time the presence of enteric viruses in surface waters of five important tributaries of Laguna Lake. This study clearly shows that enteric viruses are present in surface waters and can be subsequently used to determine fecal contamination. The high prevalence of disease-causing enteric viruses demonstrates that there is continuous fecal contamination in environmental waters. This study also shows that double filtration of water samples coupled with molecular amplification assays are suitable for water quality monitoring.

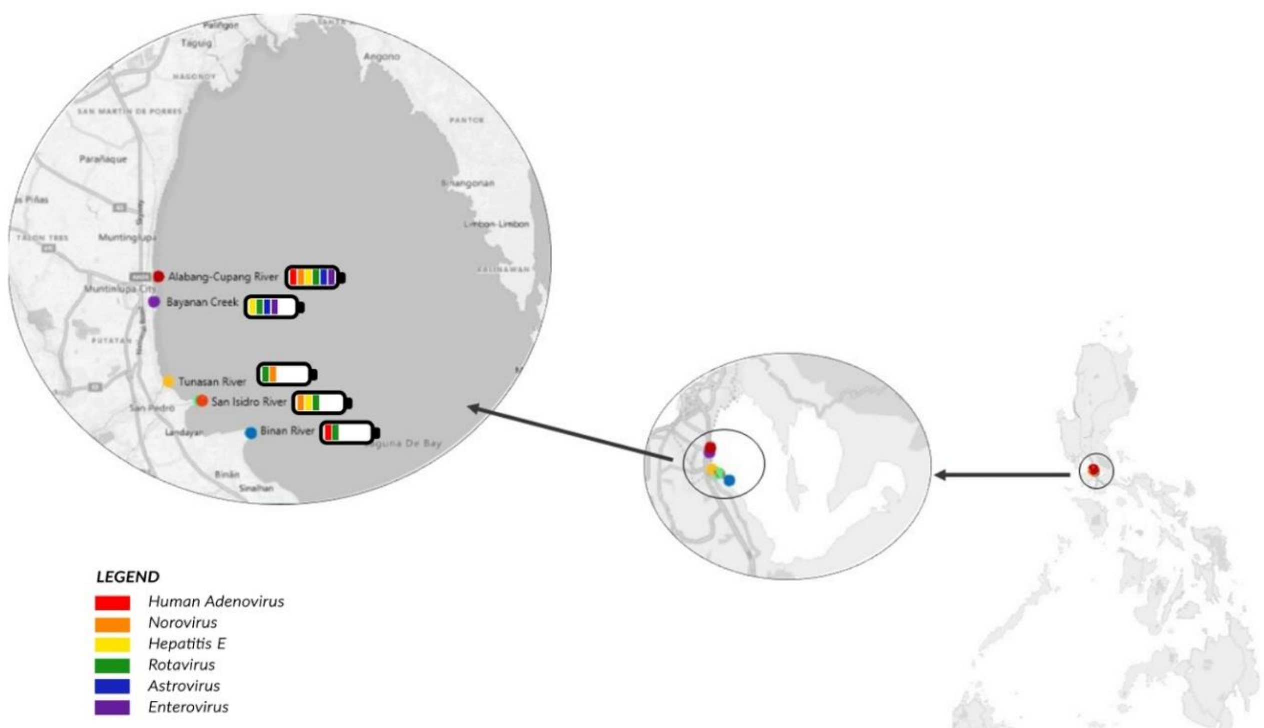

Figure 1: Map of the five sampling sites with corresponding viruses identified from each site.

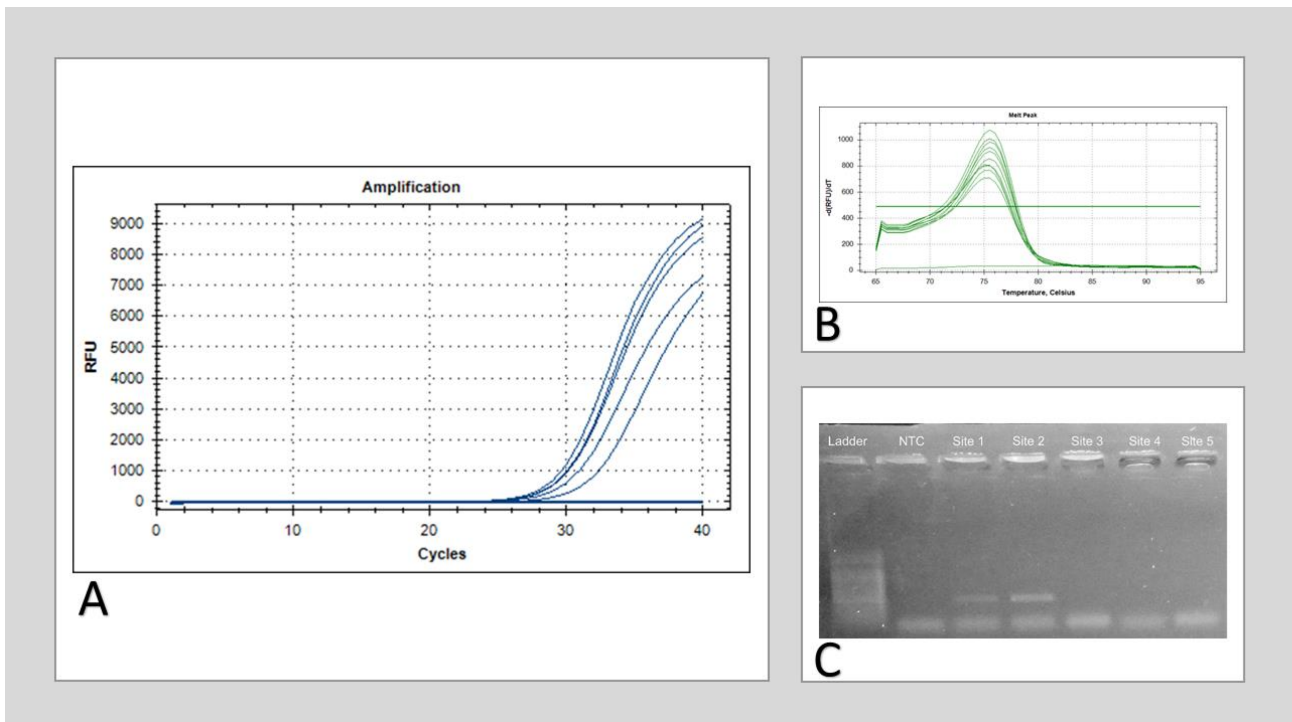

Figure 2: Representative PCR assays. (A) Amplification and (B) melt curve analysis of Rotavirus real-time PCR assays showing positive results for all five sites. Real-time PCR assays were done three times, and samples were run in duplicates. (C) Conventional PCR assay targeting the *orf2* gene of *Astrovirus* (~449 bp).

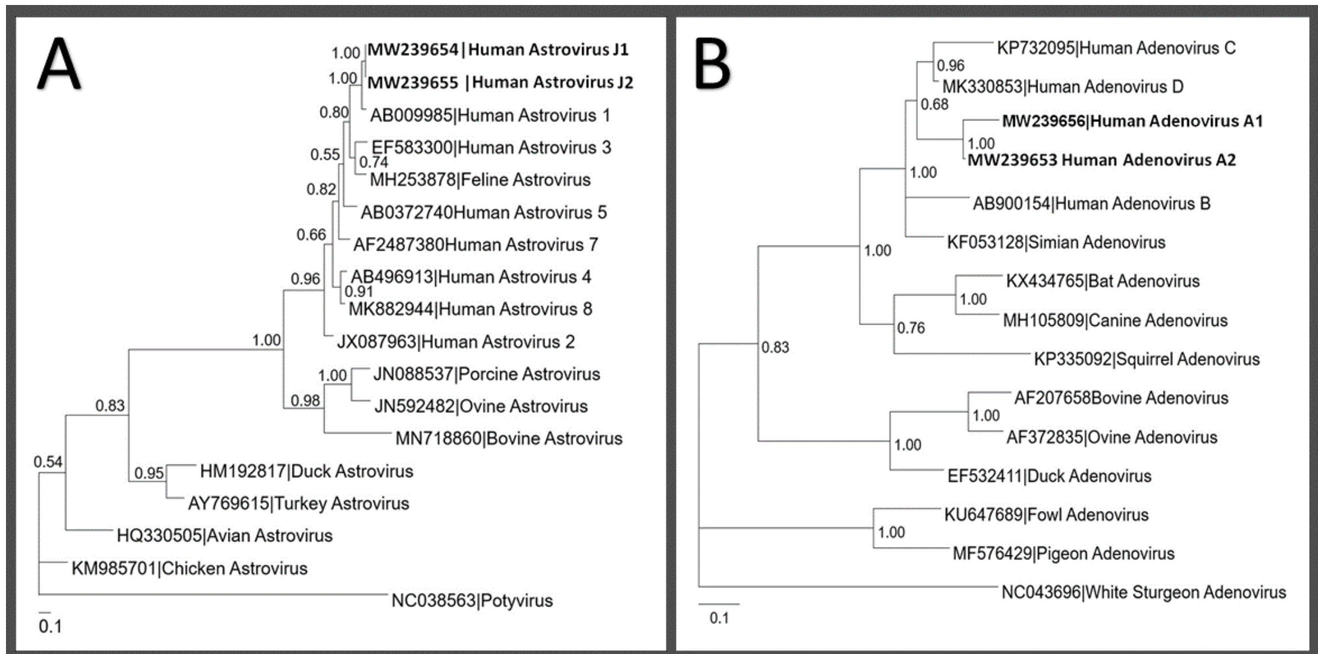

Figure 2: (A) Bayesian inference tree of samples with astroviruses (MW239654 and MW239655) with reference sequences based on 319bp *orf2* gene and two majority consensus trees. The tree is rooted on *Potyvirus* of the family *Potyviridae*. Numbers on the nodes represent posterior probabilities. (B) Bayesian inference tree of samples with adenoviruses (MW239656 and MW239653) with reference sequences based on 285bp *hexon* gene and two majority consensus trees. The tree is rooted on the White Sturgeon Adenovirus of the genus *Ichtadenovirus*. Numbers on the nodes represent posterior probabilities.

ACKNOWLEDGEMENTS

This work was financially supported by research grants from the Natural Sciences Research Institute, University of the Philippines Diliman (Project Codes: NSR-19-1-01 and NSR-20-1-03) and the Philippine Council for Industry, Energy, and Emerging Technology Research and Development (PCIEERD). We thank Christian Jordan O. Dela Rosa, Kristine Marie Flores-Destura, Mark B. Carascal, and the staff of Laguna Lake Development Authority (LLDA) for their technical assistance.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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