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Monitoring enterovirus concentration in wastewater compared with clinically confirmed case and its seasonal pattern

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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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การตรวจตราปริมาณเอนเทอโรไวรัสในน้ำเสียเปรียบเทียบกับข้อมูลผู้ติดเชื้อเอนเทอโรไวรัส

และผลของฤดูกาล

Phitchaya Kongsuwan

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บทคัดย่อ

เอนเทอโรไวรัสเป็นไวรัสที่พบได้ในแหล่งน้ำในสิ่งแวดล้อมและน้ำเสียจากโรงบำบัดน้ำเสีย มักก่อให้เกิดโรคกับเด็กที่มีอายุต่ำกว่าห้าปี ซึ่งจะก่อให้เกิดโรคมือเท้าปาก โรคทางระบบเดินอาหาร และโรคทางระบบประสาทติดต่อผ่านการอุปโภคน้ำที่มีเชื่อเอนเทอโรไวรัส จากงานวิจัยในอดีตพบว่าการระบาดของโรคมักจะเกิดขึ้นในช่วงฤดูฝน และปริมาณของเอนเทอโรไวรัสจะพบได้มากในช่วงฤดูร้อน แต่ทว่าประเทศไทยที่สภาพภูมิอากาศเป็นเขตร้อนชื้นยังมีข้อมูลของการระบาดของเอนเทอโรไวรัสที่น้อย ดังนั้นการศึกษาแนวโน้มของเอนเทอโรไวรัสในสิ่งแวดล้อมเพิ่มเติมจึงเป็นสิ่งที่สำคัญ ในการศึกษาครั้งนี้จึงได้ทำการเก็บตัวอย่างน้ำเสียจากโรงควบคุมคุณภาพน้ำสามแห่ง ดินแดง หนองแขม และช่องนนทรี ทำการเก็บตัวอย่างในช่วงระหว่างเดือนธันวาคมปี 2562 ถึงเดือนพฤษภาคมปี 2563 โดยการเก็บตัวอย่างสองครั้งต่อเดือนจากผลการทดลองพบว่าปริมาณของเอนเทอโรไวรัสในน้ำเสียไม่มีความสัมพันธ์กันในแต่ละโรงบำบัด ($p > 0.05$) นอกจากนี้ได้นำข้อมูลทางการแพทย์ของผู้ที่ติดเชื้อเอนเทอโรไวรัส จากคณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย มาหาความสัมพันธ์กับปริมาณของเอนเทอโรไวรัสจากโรงบำบัดน้ำเสีย ผลการศึกษาพบว่าไม่มีความสัมพันธ์กันอย่างมีนัยสำคัญ ($p > 0.05$) นอกจากนี้ทำการเปรียบเทียบปริมาณของเอนเทอโรไวรัสกับฤดูกาลของประเทศไทยโดยการใช้ Pearson correlation ซึ่งจากการศึกษาฤดูกาลในระยะเวลา 6 เดือน พบว่าอุณหภูมิและความชื้นไม่มีผลต่อปริมาณของเอนเทอโรไวรัส ($p > 0.05$)

คำสำคัญ: เอนเทอโรไวรัส, โรคที่เกิดจากเอนเทอโรไวรัส, ฤดูกาล

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Abstract

Enterovirus can be found in surface water and wastewater from treatment plants. It causes the disease in children under the age of five-year-old. Enterovirus infection will cause hand, foot, and mouth disease, Gastrointestinal disease, and neurodegenerative diseases. It's transmitted through the ingestion of contaminated food or water. Enterovirus outbreak usually occurs during the rainy season and the amount of enterovirus is more common during the summer. However, Thailand still has little information about the enterovirus outbreak. Therefore, it is important to further study the trends of enterovirus in Thailand. In this study, wastewater samples were collected from three treatment plants; Dindaeng, Chong nonsi and Nongkhaem. Samples were collected between December 2019 and May 2020 by sampling twice per month from the results show that the correlation between enterovirus concentration in each wastewater treatment plant is not significant ($p > 0.05$). In addition, clinical data of people infected with enterovirus case were analyzed with enterovirus concentration. The results show that there is no significance ($p > 0.05$). Also, the data of enterovirus prevalence and month was correlated to study the seasonal trend and the result shows that enterovirus did not have the specific trend in Thailand and there was not significant between temperature and humidity ($p > 0.05$).

Keywords: Enterovirus, Disease cause by enterovirus, Seasonal trend

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CHAPTER I

INTRODUCTION

1.1 Background

Nowadays, people from worldwide are paying attention to a virus outbreak, because the virus can spread in the environment and infect people rapidly. Viruses are effective when they're inside the host cell. Viruses can survive in various environments whether soil, water, or air. The survival rate of the virus in environment depends on factors such as thermal, aggregation, organic matter, and chemical. Virus has the persistence to survive and transport in several routes to another host cell by direct or indirect contact. The transmission can be through via water, aerosols, and food (Sobsey & Meschke, 2003). People contact water every day it can contain pathogenic bacteria and virus, with poor water hygiene and insanitation it causes 90% diarrheal death worldwide. World Health Organization (WHO) reported that water-transmitted viral pathogens have high health significance include norovirus, rotavirus, adenovirus, and enterovirus (Gall et al., 2015).

Enterovirus can be found in surface water, groundwater, and sewage. It is a non-enveloped single-stranded RNA virus within the Picornaviridae family, small size, and lack essential lipids (Wells & Coyne, 2019). Enterovirus has been reported it mostly affected children younger than 5 years old (Rao, 2020), causing various diseases such as hand, foot, and mouth disease, herpangina, gastrointestinal diseases, polio, and neurological disease. The range of symptoms is fever to fatal and the transmission is from consuming contaminated water. Some disease can infect through respiratory routes (Zaoutis & Klein, 1998). Thus, the waterborne disease caused by enterovirus has been seriously concerned.

Enterovirus caused various diseases. Gastrointestinal disease is one of the diseases that cause by an enterovirus, because enterovirus is tolerant in acidic pH and higher temperature and can survive in the human gastrointestinal tract (Zaoutis & Klein, 1998) that lead to gastrointestinal disease in people. It caused 499,000 death annually in children below 5 years old (Rao, 2020). Hand, foot, and mouth disease mostly infected in children, the symptom is a rash on hand, foot, and mouth and with some fever (Muehlenbachs et al., 2015). To know the trend of people infection, age-range and sex of patients that have disease from enterovirus is collect in database and use for study trend of enterovirus infection case in each year. The epidemiological study of enterovirus disease in the community can be done through the collection of clinical cases from various hospitals in a different area. The disease occurrence and spread can be understood from this data. Nevertheless, clinical data cannot collect the number of patients who have the asymptomatic disease. So, the data of disease caused by enterovirus will not collect in the database. Thus, to check the true number of enterovirus infections it can be done through virus surveillance in the environment.

Wastewater-based epidemiology (WBE) is used to study the prevalence of enterovirus in the environment and surveillance community health by detecting virus in fecal from wastewater at the municipal wastewater treatment plant. The WBE can collect the data of asymptomatic disease by collect the number of viruses that are directly shed into wastewater. The integration of clinical data and WBE can increase the knowledge of enterovirus epidemiological and can predict the trend of enterovirus outbreak.

The prevalence of enterovirus also depends on factors, such as pH, humidity and precipitation. Seasonality is one of the important factors of the circulation and prevalence of enteroviruses in the environment. From the previous study, it showed that enterovirus usually highly presented in summer and low levels in winter and spring (Hsiao, 2012). With this information, the period of routine enterovirus outbreak can be predicted. However, Thailand is in a tropical zone and still lacks of data of the prevalence of enterovirus in the water environment and its seasonal effect. If the relationship between the prevalence of enterovirus in environment and the season in Thailand can be determined, it can be the new data for the circulation of enterovirus in Thailand.

Overall, the objectives of this research are to investigate the correlation between the prevalence of enterovirus from the wastewater treatment plant and disease caused by an enterovirus and to determine the correlation between the seasonality and the prevalence of enterovirus in Thailand.

1.2 Objective

1.To determine the correlation between the prevalence of enterovirus from the wastewater treatment plants and the number of patients caused by enterovirus.

2.To study the seasonal effect on the prevalence of enterovirus in wastewater.

1.3 Benefits

1.The result from this study can predict the trend of disease caused by enterovirus by considering the concentration of enterovirus in wastewater.

2.The result will give new information on seasonality and the circulation of enterovirus in Thailand.

3.The result gives a better understanding of enterovirus prevalence in wastewater

CHAPTER II

THEORETICAL BACKGROUNDS AND LITERATURE REVIEW

2.1 Enterovirus background

Enterovirus is from the family *Picornaviridae*, non-enveloped and single-stranded RNA viruses (Muehlenbachs et al., 2015). It was first isolated in California, USA, in 1969 from an infant that has encephalitis (Linsuwanon et al., 2014). The receptor of enterovirus is found on white blood cells and the gastrointestinal tract (Solomon et al., 2010). The infection mostly occurred during the summer months and epidemics are correlated with a seasonal pattern (Solomon et al., 2010).

Enterovirus caused various diseases such as hand, foot, and mouth disease, herpangina and gastrointestinal disease that mostly affects children younger than 5 years old (Solomon et al., 2010). Moreover, the recent study shows that age has a great association with disease: central nervous system disease mostly occurs in age 5-15 year old, in 20-40 year old the most infectious disease is myocarditis (Muehlenbachs et al., 2015). Enterovirus also associated with brainstem encephalitis, leading to fatal cardiopulmonary collapse, and a severe case of acute myelitis, leading to long-term paralysis (Messacar et al., 2020). It also caused an important emerging viral encephalitis in Southeast Asia (McMinn, 2012). The disease that mostly concern from enterovirus is poliomyelitis. Therefore, the organization from the worldwide are collaborated to eradicate this disease from the world and the result is polioviruses is mostly eliminated from many parts of the world except for the horn of Africa, Pakistan, and Syria (Muehlenbachs et al., 2015).

The route of enterovirus transmission is by the fecal-oral route, through water and food that contaminated, or respiratory track (Wieczorek et al., 2015) The range of symptoms is fever to fatal. Enterovirus can be found on the surface and groundwater, they can survive at room temperature for several days in the environment and can be inactivated in temperatures higher than 56 ^{o} C, chlorination, formaldehyde, and ultraviolet irradiation (Solomon, Lewthwaite, Perera, Cardosa, et al., 2010).

To prevent enterovirus infection, a vaccine is required. However, vaccine is not currently available for enteroviruses (McMinn, 2002) because there is still some obstacle that make enterovirus vaccine doesn't fully complete. The lack of a suitable animal model for the vaccine development makes enterovirus vaccine are ongoing development (McMinn, 2012).

2.2 Enterovirus seasonality

Temperature and relative humidity is the factor that can affect the persistence of enterovirus and transmission in the environment. Enterovirus presence in Asia was tend to peak in summer (Hsiao, 2012). The study in the Europe region from Wieczorek et al., 2015 that studies the circulation of non-polio enterovirus in sewage in Poland and the result shows that it had higher rate in summer and autumn. Meijer et al., 2014 also show that enterovirus continued to circulate in a seasonal pattern. Overall from the previous research in Europe imported that enterovirus was mostly found in summer. However, Thailand is in the tropical zone that still lacks enterovirus seasonality data. Thus, to determine the relationship between the season in Thailand and the presence of enterovirus will be the new database. The estimation of the peak of enterovirus in each month all year round can be used to predict the trend of enterovirus pandemic in the environment.

2.3 Diseases caused by enterovirus

Diseases caused by enterovirus is a public concern that transmission through various route causes hand, foot, and mouth disease, herpangina, poliovirus, and gastrointestinal disease. A recent study shows that the transmission of enterovirus is from contact with an infected sibling and the transmission rate is 84 % (Lee et al., 2009). Gastrointestinal disease is one of the diseases caused by enterovirus it caused diarrhea, mortality, and morbidity. From the previous study, enterovirus mostly infected in children younger than 5 year old caused 1.6 million deaths annually (Rao, 2020). Gastrointestinal disease transmitted through the fecal-oral route and tends to present in poor hygiene areas and during summer (Zaoutis & Klein, 1998). Hand, foot, and mouth disease epidemics have associated with coxsackievirus and herpangina (Lee et al., 2009). Mostly infection in young children causing illness and death (Dong et al., 2007). The symptom is rash on the anterior hand, foot, and mouth, also associated with mild viral illness (Muehlenbachs et al., 2015).

2.4 Environmental surveillance

Environmental surveillance is the detection of virus circulation in the environment. With research application, special laboratory skills, and sustainable field will help environmental surveillance have more effective (WHO, 2003). The recent study it show that environmental surveillance is important in helping poliovirus eradicate, it helps detect viruses before paralytic cases are reported (Hamisu et al., 2020). Enterovirus comes from fecal that people shedding into the environment and sewage it remains in several weeks in the environment (Kargar et al., 2009). Environmental surveillance can show the number of enterovirus circulation in the sewage and environment. Brinkman et al., 2017 studied the surveillance enterovirus in municipal wastewater for 1 year. The result showed that enterovirus concentration ranged from 3.8 to 5.9 log_{10} equivalent copies/liter in monthly samples and can be estimated that 2.8% of enterovirus was shedding from the population in daily.

2.5 Microbial indicator

Microbial indicator was used for estimated public health risk in recreational water and efficiency of water treatment plant (Wu et al., 2011). The microbial indicator has various species such as *E. coli*, total coliforms, F-specific coliphages, and bacteriophage. Microbial indicator also used to be a surrogate for the pathogen because they share many fundamental properties, features, structure, composition, and morphology with the same of viruses (Grabow, 2001). Furthermore, compared with the viruses, bacteriophage is easy to be detected and monitored in the environment and have high number in wastewater and rapidly cultivated in the laboratory, and used low-cost techniques. Besides, the previous study in brazil found that the correlation between coliphage and the prevalence of enteric viruses were correlated (Dutka et al., 1987) and *E. coli* significantly associated with gastrointestinal illness in the population (McNeill, 2019). However, some study shows that some factors were affecting the correlation between microbial indicator and pathogen include individual organism resistance to environmental and growth, carriage rates and shedding patterns among populations (Wu et al., 2011) and microbial indicator and viruses do not enter wastewater with the same ratio and they have different survival rates (Wyer et al., 1995). Furthermore, the recent study showed that coliphages concentration may increase under suitable environmental conditions (Stetler, 1984). Also, virus concentration in water and wastewater were depend on rainfall and salinity (Gerba et al., 1979). Thus, this studied the relationship between microbial indicators that are *E. coli* and bacteriophage and enterovirus concentration in the wastewater treatment plant.

2.6 Wastewater-based epidemiology

Wastewater-based epidemiology (WBE) has been used to examine emerging chemicals and drug use pattern from influent in wastewater treatment plant (Gonzalez et al., 2020) and it is the tool for assessing the presence of the virus spreading in the community by detecting the pathogens in fecal from people that shedding into the water and collected sample from the municipal plant. It has an efficient approach with great potential for early warning of infectious disease transmission and outbreaks (Sims & Kasprzyk-Hordern, 2020). WBE also combined techniques related to the extraction, analysis, data processing, and interpretation of targets excreted from feces in wastewater, which give comprehensive community health information on the spread of the virus in specific population (Venugopal et al., 2020). WBE can detect asymptomatic disease in the community because WBE has the potential to identify both symptomatic and asymptomatic disease (Gonzalez et al., 2020). Thus, with WBE it can help collect the true number of patience virus infection case in the community and WBE also have hypothesis that can give a reflection of the population health in (near)-real-time (Sims & Kasprzyk-Hordern, 2020).

WBE is integrated with a clinically confirmed case, combined with virus concentration in environment or wastewater and number of the clinically confirmed case it can be the early warning of a virus outbreak in the community. The recent study, showed that Covid-19 that is the virus that first outbreak in late 2019 was used WBE and clinical confirmed case to observe the trend and give more understanding of outbreaks of Covid-19 (Gonzalez et al., 2020). Furthermore, McCall et al., 2020 was detected picornaviruses in untreated wastewater by used WBE for identification upcoming viral outbreaks. The results showed that WBE has the potential to approach the area of disease outbreak and can improve public health.

2.7 Virus concentration and detection in water environment

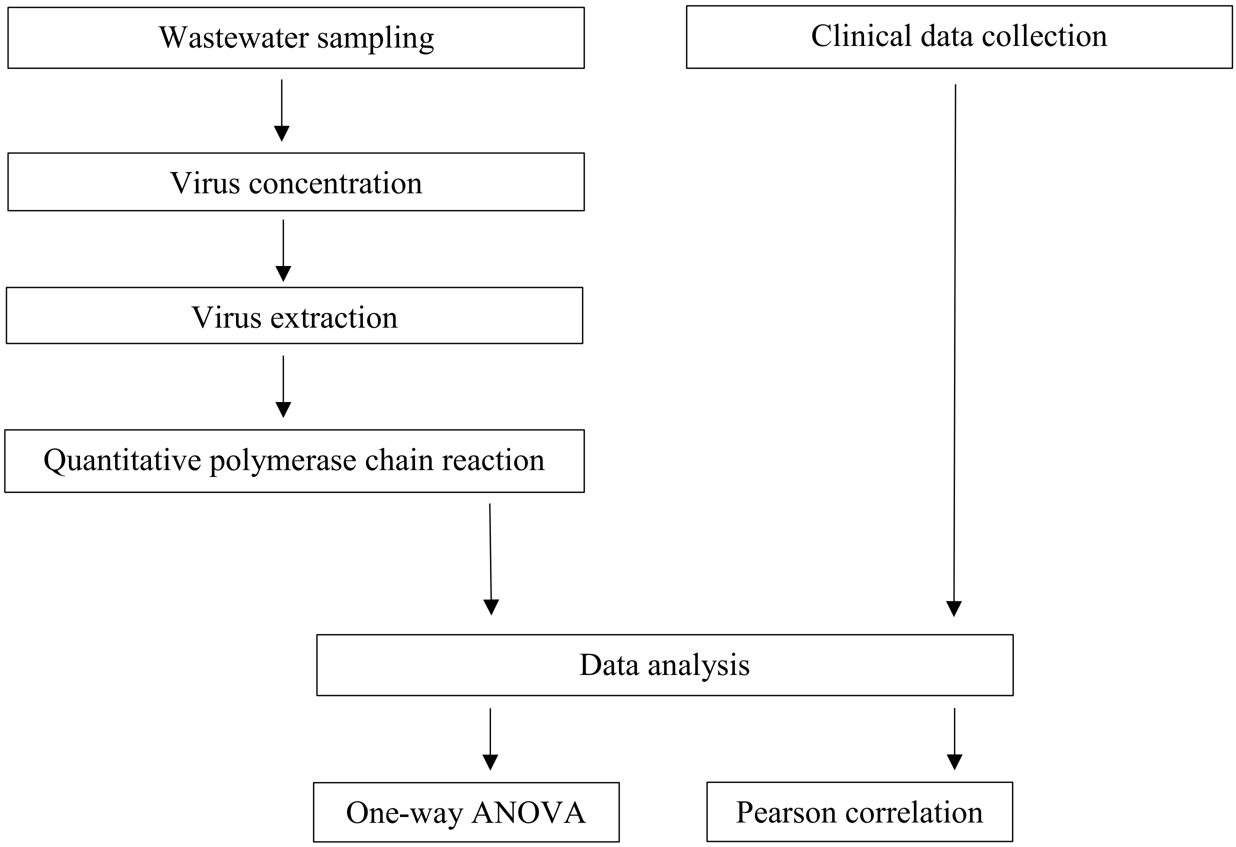
Prevalence of enterovirus in the environment, virus is highly diluted in wastewater and have some factor affect the virus concentration that makes it difficult to be detected directly. Thus, the virus concentration method is crucial to increase its concentration. By selecting the method that appropriate for the virus type, it will increase the virus detection efficiency in the water sample. One of the virus concentration methods is the electronegative membrane technique which used the difference of charge between virus and membrane (Katayama et al., 2002). Normally virus is a negative charge when the solution is mix into the water sample that have virus it will change the charge of the virus into the positive charge that makes virus can attach to the membrane and elute the virus from the membrane by a solution such as NaOH.

RT-qPCR analysis was widely used for detecting the virus in a water environment due to its sensitivity and specificity. Viral RNA will be reversed to complementary DNA (cDNA) in the reverse transcription process, and then cDNA will be amplified during qPCR process. This will make the virus have plenty amount of virus for detection. Thus, RT-qPCR analysis was used to detect DNA viruses in the water sample. From recent study, RT-qPCR assays was used to detected on norovirus, human, adenovirus and hepatitis A virus present in sewage (McCall et al., 2020).

CHAPTER III

MATERIALS AND METHODS

3.1 The overall processes



3.2 Research Material

3.2.1 Laboratory Instruments

1. MSE Falcon 6/300 Large bench centrifuges
2. Precisa 2200 CSCS Balances, Scales, and Weighing
3. Hirayama HVA-85 Autoclave
4. Sanden Intercool Freezer
5. Argo Refrigerator
6. GAST RAA-V110-ED Pumps
7. Biosafety cabinet

8. QuantStudioTM 6 Flex Real-Time PCR System
9. QIAamp Viral RNA Mini Kit
10. Pipette 10 ml
11. Vacuum pump
12. Filter flask
13. Filter membrane
14. Funnel
15. Micropipette tips
16. Erlenmeyer flask
17. Measuring cylinder
18. Micro-centrifuge tube
19. Forceps
20. Bottle
21. Beaker
22. Glass Petri Dish
23. Cylinder
24. Aluminum Foil
25. Autopipette
26. PCR Tube
27. Vortex-Genie 2

3.2.2 Chemical reagents

1. $MgCl_2$
2. H_2SO_4
3. 1 mM NaOH

4. 100x Tris-EDTA buffer

5. Enterovirus spike

6. Enterovirus primer

7. RNA extraction kit

8. Probe

9. Ethanol (96-100%)

10. Taq Polymerase

3.3 Wastewater sampling

Influent and effluent wastewater were collected twice a month from December 2019 until May 2020 by grab sample method from three wastewater treatment plants, Dindaeng, Chongnonsi, and Nongkhaem.The water sample is collected in the bottle each 1L and store in a container approximately at 8 ^{o} C. The data of temperature and humidity wasn't collect during wastewater sampling. Using temperature and humidity that average in each month and the data is collected in Bangkok area from the database from Tutiempo Network, S.L. 2021, https://en.tutiempo.net/climate/01-2020/ws-484550.html.

3.4 Clinical data

The number of enterovirus infection cases and hand, foot, and mouth disease was obtained from the center of Excellence in Clinical Virology that collects the data from four hospitals, King Chulalongkorn Memorial Hospital, Thonburi 2 Hospital, Phyathai 3 hospital and N Health with the number of enterovirus infection case. The data is collected from December 2019 until May 2020. All of the four hospitals are in Bangkok.

3.5 Virus concentration

Increase virus concentration in wastewater sample by added 5.3 g of $MgCl_2$. 400 ml of influent wastewater sample or 800 ml of effluent wastewater sample were poured into the membrane. 0.5nM H_2SO_4 200 ml was used to rinse the membrane. The centrifuge tube was attached to a vacuum pump set containing 50 μl of 0.5 mM H_2SO_4 and 100 μl of 100x TE buffer, then 10 ml of 1 mM NaOH are rinsed virus into the tube. virus concentration in a water sample by secondary concentration method, the sample is poured into Amicon® Ultra – 15 tubes and centrifuge by using MSE Falcon 6/300 Large bench centrifuges at 6000 rpm for 15 minutes, note the final water sample that remains in Amicon® Ultra – 15 tubes.

3.6 Virus Extraction

QIAgen® viral RNA extraction kit (Qiagen) was used in the virus extraction method. 140 μl water sample was pipetted into a microcentrifuge tube. The volume of Buffer AVL and carrier RNA-AVE are prepared depending on the number of samples. Mix the reagent by pulse-vortexing and incubate at room temperature for 10 min. After that, 560 μl of ethanol is pipette into the microcentrifuge tube and mix the reagent again by pulse-vortexing. Move the sample 630 μl to the QIAamp® Mini column and centrifuge at 8000 rpm for 1 min, repeat the step until remove all of the water samples in a microcentrifuge tube. 500 μl Buffer AW1 was add and centrifuge at 8000 rpm for 1 min. QIAamp® Minicolumn was placed in a clean 2 ml collection tube and add 500 μl Buffer AW2 centrifuge at full speed for 3 min. The QIAamp® Mini column was placed in a clean microcentrifuge tube and add 60 μl Buffer AVE equilibrated and incubate at room temperature for 1-minute and Centrifuge at 8000 rpm for 1 min. Then remove the QIAamp® Mini column and collect the final water sample in a microcentrifuge tube.

3.7 Quantitative polymerase chain reaction (qPCR) assays

TapMan Fast Virus 1-Step Master Mix (Thermo Fisher) was prepared in a biosafety cabinet with the reagent in **Table 1.** The sequence of the primers used for detection and quantification of enterovirus was shown in **Table2**. The overall volume of the master mix is prepared by multiplication each volume reagent with the number of the sample that tested. Then, 8 μL of the reagents master mix and 2 μL sample was pipetted into a microcentrifuge tube. The microcentrifuge tube is placed on PCR rack in the QuantStudioTM 6 Flex Real-Time PCR with the condition in **Table 3**. The result will show in amplification curves.

Table 1 Master mix preparation

Reagents	Volume per reaction (μl)
4X TaqMan Fast Virus	2.5
10 M Forward Primer	0.4
10 M Reverse Primer	0.4
10 M Probe	0.3
DEPC H_2O	4.4
Total	8.0

Table 2 Sequences of the primes and probe name

Primer / Probe name	Sequence (5’ -> 3’)
EV-5’UTR-qF (Forward primer)	TCCTCCGGCCCCTGAATG
EV-5’UTR-qR (Reverse primer)	ATTGTCACCATAAGCAGCCA
EV-5’UTR-probe	FAM-GCAGCGGAA/ZEN/ CCGACTACTTT-Iowa Black FQ

Table 3 qPCR condition

PCR steps	Temperature	Time	Number of cycles
Reverse transcription	50 ^{o}C	5 min	1
RT–inactivation/Initial denaturation	95 ^{o}C	10 min	1
Denaturation	95 ^{o}C	15 sec	45
Annealing/extension	60 ^{o}C	1 min	

3.8 Statistical analysis

IBM SPSS software is using for analyzing the statistic. One-way ANOVA is selected for the relationship between the enterovirus concentration in each wastewater treatment plant. The correlation between the number of enterovirus infection cases and enterovirus concentration is using Pearson correlation and it is also used for determining the correlation between temperature, humidity, and the concentration of enterovirus from the wastewater treatment plant for seasonality trend.

CHAPTER IV

RESULT AND DISCUSSION

4.1 Enterovirus prevalence

Enterovirus was detected from three wastewater treatment plant from each influent and effluent. The concentration of enterovirus in each wastewater treatment plant are shown in **Table 4**. Dindaeng wastewater treatment plant had positive results only in December 2019. The enterovirus concentration in influent was 0.322 MPN/ml and in effluent was 0.053 MPN/ml. For Nongkhaem wastewater treatment plant, it had only positive result from influent, that was in January 2020, February 2020, and May 2020 with the concentration 0.010 MPN/ml, 0.167 MPN/ml, and 0.017 MPN/ml. Chongnonsri wastewater treatment plant had positive result in December 2019. The concentration in effluent was 0.059 MPN/ml, January 2020 the concentration in influent was 0.470 MPN/ml and effluent was 0.238 MPN/ml, February 2020 the concentration in effluent was 0.095 MPN/ml and March 2020 the concentration in influent was 0.149 MPN/ml. The symbol of " - " that showed in the table indicated the value below detection limit of 0.021 MPN/ml. The statistical analysis didn't used the value that was below 0.021 MPN/ml into calculated. Thus, the enterovirus concentration from Nongkhaem with the concentration 0.010 MPN/ml and 0.017 MPN/ml didn't used in analyzed. Average enterovirus concentration is from the two samplings once in two weeks the data value is from the PCR analysis and then using reverse calculation to get the first concentration of virus in wastewater.

The result showed that the detection rate of enterovirus from Chongnonsi wastewater treatment plant (CH) was higher than that from Nongkhaem wastewater treatment plant (NK), and Dindaeng wastewater treatment plant (DD), respectively. One-way ANOVA is used for analyzed the relationship between enterovirus concentration in each wastewater treatment plant. The p-value between wastewater influent is 0.387 which shows non-significant (ANOVA, $p > 0.05$), in the same way with the result from wastewater effluent that shows the p-value of 0.142 mean it is non-significant (ANOVA, $p > 0.05$). The reason described that the enterovirus concentration value that used in analysis is less, mostly it uses detection limit to analyze which makes it is not various of value.

From the result in this study, it showed that enterovirus could be detected from wastewater in Thailand. Enterovirus was also reported found in wastewater from wastewater treatment plants in the United states, Simmons & Xagoraraki, 2011 were collected 30 wastewater and 6 biosolids samples from five wastewater treatment plants and found 67% of the enterovirus in the wastewater samples. Katayama et al., 2008 surveyed the enterovirus from 72 samples in influent and effluent from 6 wastewater treatment plants in Japan and the result showed that enterovirus was found in the effluent and influent with 17 RT-PCR units/ml and 0.044 RT-PCR units/ml respectively.

Table 4. The enterovirus concentration in each wastewater treatment plant in month

Month / Year	Wastewater Type	Enterovirus Concentration (MPN/ml)		
		Dindaeng	Nongkhaem	Chongnonsri
December 2019	Influent	0.322	-	-
	Effluent	0.053	-	0.059
January 2020	Influent	-	-	0.470
	Effluent	-	-	0.238
February 2020	Influent	-	0.167	-
	Effluent	-	-	0.095
March 2020	Influent	-	-	0.419
	Effluent	-	-	-
April 2020	Influent	-	-	-
	Effluent	-	-	-
May 2020	Influent	-	-	-
	Effluent	-	-	-

Note: “ – ” symbol indicates the result below the detection limit

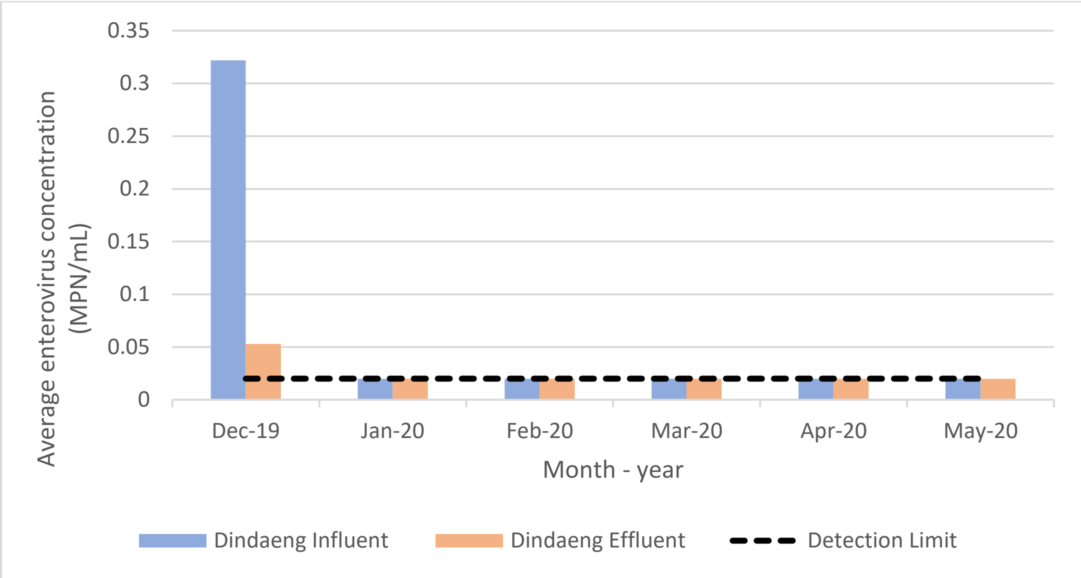


Figure 1. Enterovirus concentration from Dindaeng wastewater treatment plant

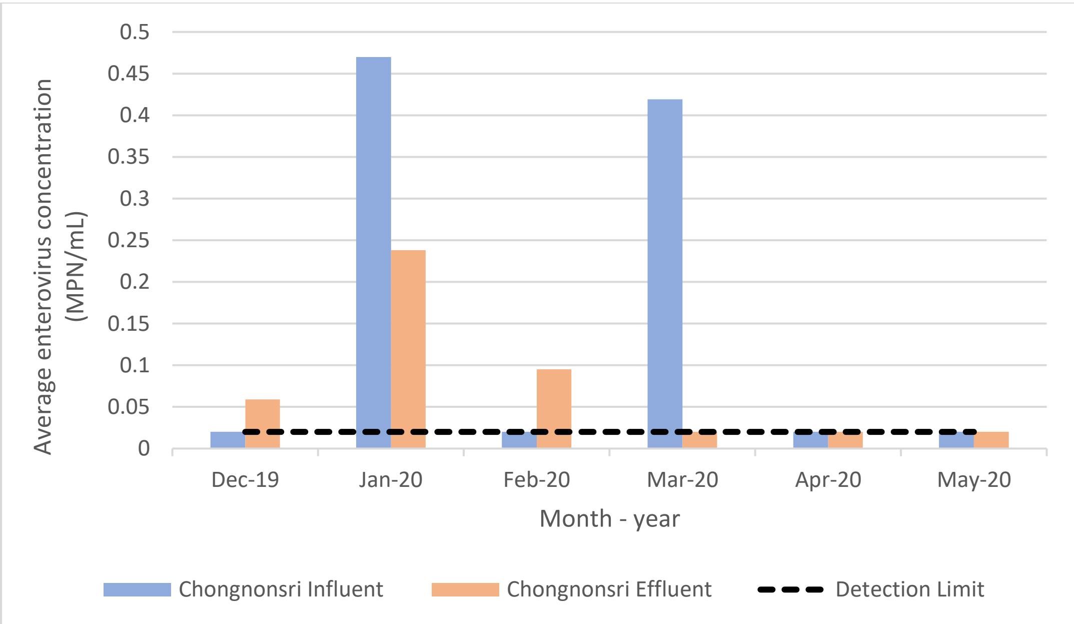


Figure 2. Enterovirus concentration from Chongnonsri wastewater treatment plant

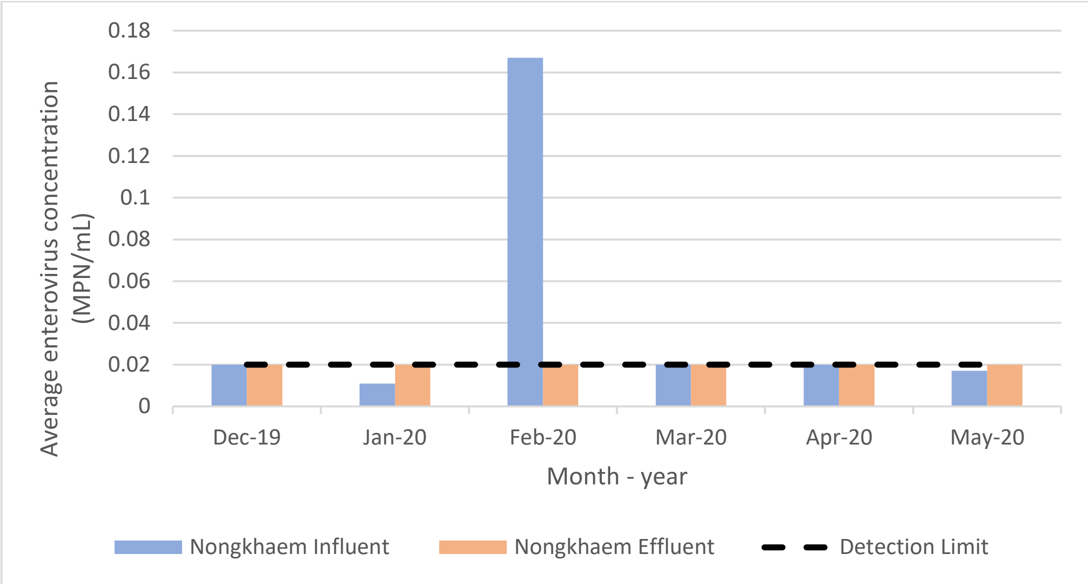


Figure 3. Enterovirus concentration from Nongkhaem wastewater treatment plant

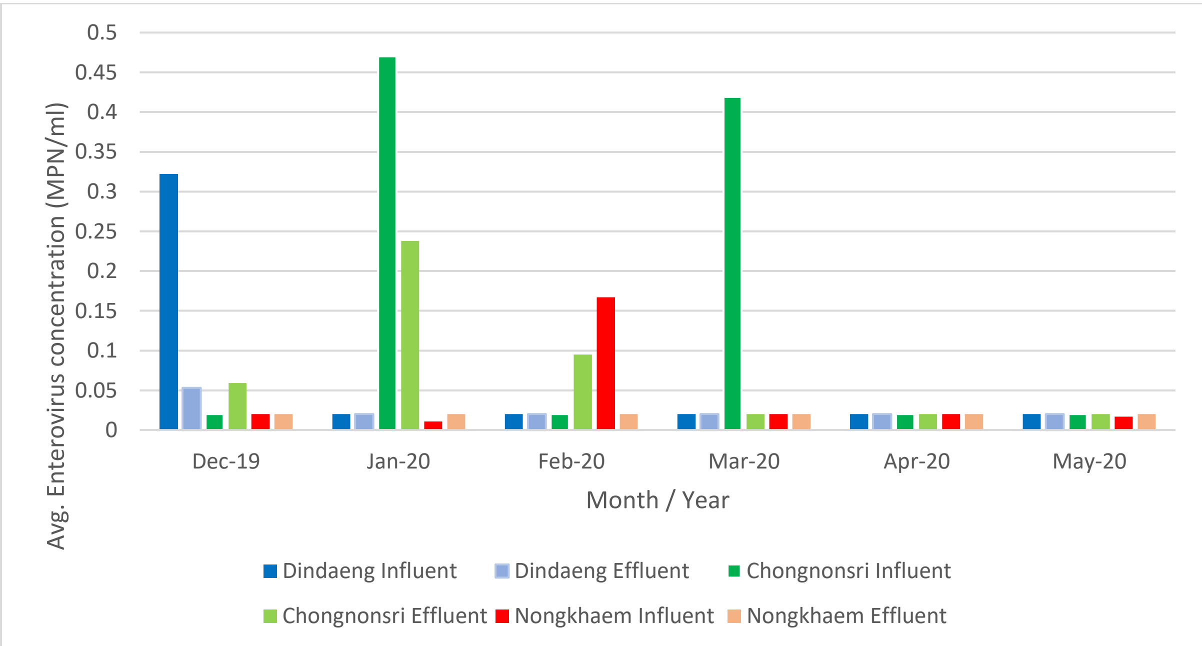


Figure 4. Trend of average enterovirus concentration in month from each wastewater treatment plants

4.2 Enterovirus and the correlation with microbial indicator

Bacteriophage and *E. coli* were a microbial indicator that can be used to predict the presence of enterovirus in the environment because they has removals efficiency in wastewater treatment plant and has resistant like enterovirus (Stetler, 1984). However, further study of this relationship was still required. That conduce to this adjunct study in this research to enhance the information for bacteriophages and *E. coli* as an indicator of enteroviruses by bringing microbial indicator concentration data from Tantai, 2020. If bacteriophage and *E. coli* have a correlation with enterovirus with prevalence and concentration in the same way, this microbial can be used an indicator for enterovirus.

Enterovirus concentration and microbial indicator data were collected during January 2020 to March 2020. The enterovirus concentration was used only from influent sample with highest concentration in January from Chongnonsri wastewater treatment plant with 0.470 MPN/ml while bacteriophage and *E. coli* concentration were highest in January from Nongkhaem wastewater treatment plant with 10500 CFU/ml and 168 PFU/ml respectively. Enterovirus and microbial indicator were correlated by using Pearson correlation the result showed that with enterovirus and bacteriophage was not significance ($p = 0.426$) and between enterovirus concentration and *E. coli* also not significance ($p = 0.569$). The reason was bacteriophage and *E. coli* were bacterial flora that always living in the general people but enterovirus is only living in the person that gets enterovirus infection. Thus, the amount of enterovirus shedding into the wastewater treatment plant from people can be less number than bacteriophage and *E. coli*, that always shedder from people into the wastewater every day. Furthermore, the previous study from Stetler, 1984 reported that bacteriophage concentration in the environment may increase under the suitable condition that make bacteriophage concentration are more than enterovirus. Moreover, Wu et al., 2011 showed that shedding patterns among the populations can affect the correlation between microbial indicator and viruses.

4.3 Enterovirus seasonal trend in Thailand

Enterovirus concentration in the environment and wastewater can be effect by various factors. For example, seasonality was one of the factors that can affects enterovirus prevalence in the envrionement due to the different in temperature and humidity in each season that leads to the difference of enterovirus prevalence. The enterovirus concentration in influent was collect during December to May from three wastewater treatment plants. The temperature and humidity information were from the collected database in Bangkok area and the data of temperature and humidity were average in each month.

Average enterovirus concentration from three wastewater treatment plant in each influent was showed in **Table 5.** January 2020 had the highest enterovirus concentration with 0.167 MPN/ml. Pearson correlation were used for statistical analysis and the result showed that there is no correlation between enterovirus concentration and temperature ($p = 0.207$), and enterovirus concentration and humidity there ($p = 0.988$). Thus, the study showed that enterovirus concentration was no relationship with temperature and humidity and cannot specify the seasonal trend of enterovirus in Thailand.

The relationship between temperature and enterovirus concentration is represented in **Figure 5**. It showed that temperature tended to increase from December to May. From **Figure 6**., the humidity tends to increase in early March but decreases in April and May. When compare enterovirus concentration with temperature and humidity the result showed that enterovirus concentration in each month there was no explicit trend, in December and January the concentration tended to increase but in February the concentration was decreased and increase again in march and rapidly decrease in April and May that be summer season in Thailand. On the other hand, the previous study from Pons-Salort et al., 2018 was investigated enterovirus in United states and the result showed that enterovirus concentration was highest in summer, with similar to the study from Kumthip et al., 2017 that found enterovirus detection rate was increase in summer. The reason might be that the season in Unites states had clearly different changing in each season that the average temperature in summer is 34 ^{o} C and winter is -12 ^{o} C. However, the weather in Thailand was not clearly different in each month, the average temperature in summer is 35 ^{o} C and winter is 26 ^{o} C, that can be one of the factors why enterovirus trend in Thailand and United states were different. Besides, Thailand has three seasons that are summer, rainy and winter. Summer in Thailand was during February to May but in United states have four season that is summer, winter, fall, and spring it does not have rainy season and summer in United states was during June to August because of this different season pattern, in order to specify the trend prevalence of enterovirus it must specify in season. However, overall results from this research were based on data collection in 6 months. To get the better of information, the research in all year round can make the proper information about enterovirus seasonal trend in Thailand.

Table 5. Average enterovirus concentration from influent in each wastewater treatment plant

Month / Year	Average enterovirus concentration (MPN/ml)
December 2019	0.121
January 2020	0.167
February 2020	0.070
March 2020	0.153
April 2020	-
May 2020	-

Note: " – " symbol indicates the result below the detection limit

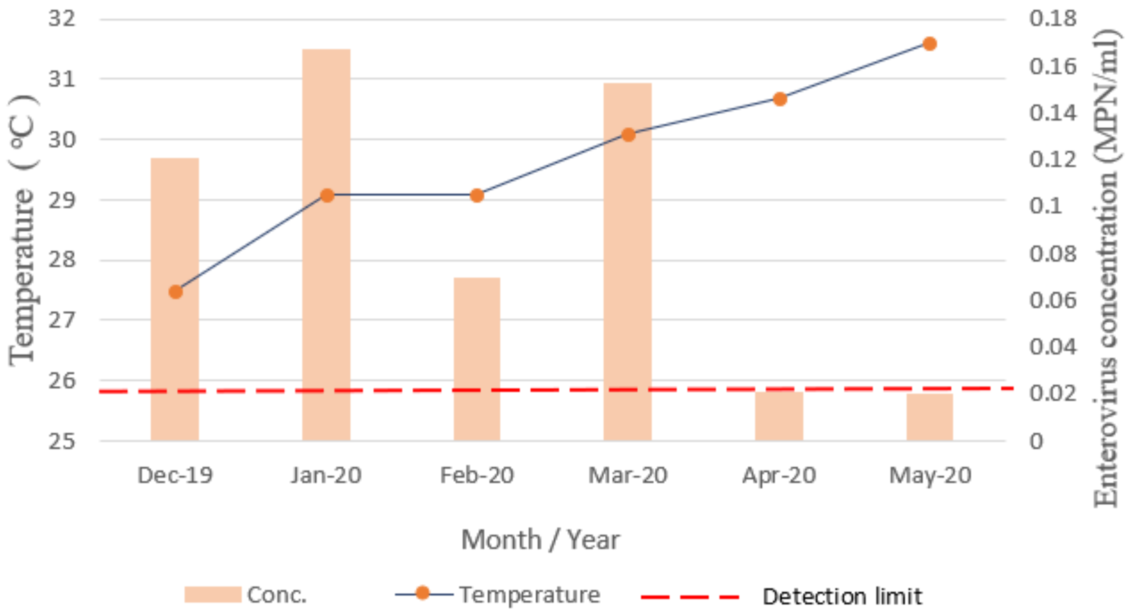


Figure 5. Enterovirus concentration compared with temperature

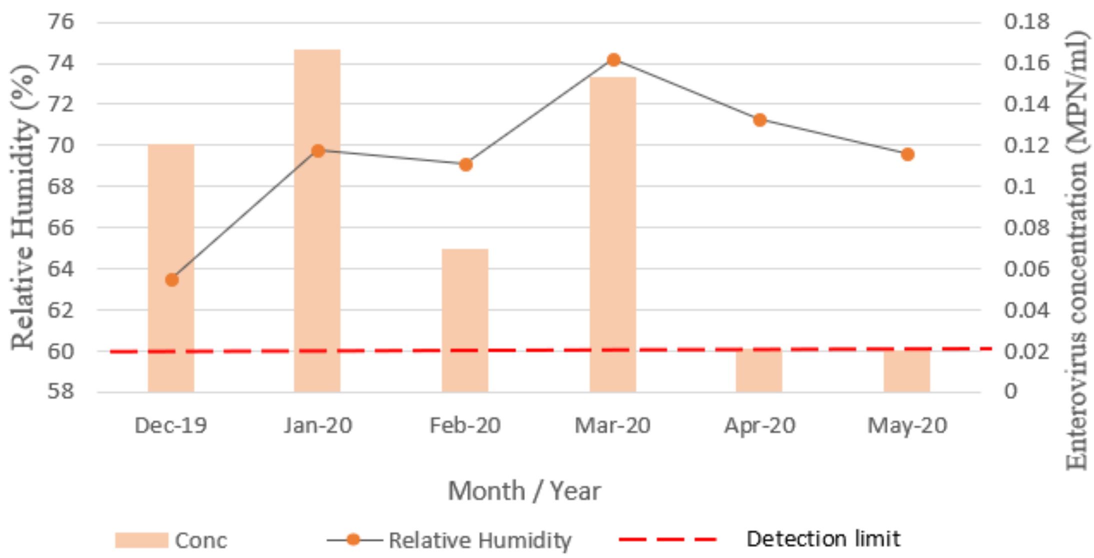


Figure 6. Enterovirus concentration compared with relative humidity

4.4 Enterovirus and clinical data correlation

The Clinical data was from the database of four Hospitals (King Chulalongkorn Memorial Hospital, Thonburi 2 Hospital, Phyathai 3 Hospital, and N Health). The cases were the disease caused by enterovirus and hand, foot, and mouth disease were all included in the number of cases that uses for statistical analyzes. The number of cases and average enterovirus concentration in influent from three wastewater treatments plant was represented in **Table 6**. It showed that January was the month that had the most patient with 9 cases while in March, April and May weren't found. Enterovirus concentration was highest in January 2020 with 0.167 MPN/ml.

In **Figure 7**, it represents that in January 2020 the number of case and enterovirus concentration was correlated, while another month the number of cases was less than enterovirus concentration. The reason might be patients didn't admit in hospital. So, the information was not collected in the database. In order to increase the accurate information, the increase of several enterovirus infection cases from the different hospital and more cover area is needed. The correlation between enterovirus concentration and clinical data is analyzed by Pearson correlation, the result showed that they were not correlated ($p > 0.05$).

Comparing the results with the previous study from Puenpa et al., 2018 that was studied enterovirus infection in Thailand throughout the year, Puenpa et al., 2018 found that enterovirus infections increased frequency in the rainy season (June-September). However, this study wasn't shown the obvious trend of enterovirus infection cases. One of the reasons was that the trend of enterovirus infection cases couldn't be specified was that the data of enterovirus infection was collected during the first wave of Covid-19 infection in Thailand. So, people tended to stay home to prevent from COVID-19 infection. The patient that has enterovirus infection might not go to hospital and make the data of enterovirus infection case in patience was not complete. Also, this study only proceeded in 6 months and it didn't show the trend in all year-round.

Table 6. Number of Enterovirus infection case in each month

Month / Year	Enterovirus concentration (MPN/mL)	Number of case
December 2019	0.121	1
January 2020	0.167	9
February 2020	0.070	6
March 2020	0.153	0
April 2020	-	0
May 2020	-	0

Note: " – " symbol indicates the result below the detection limit

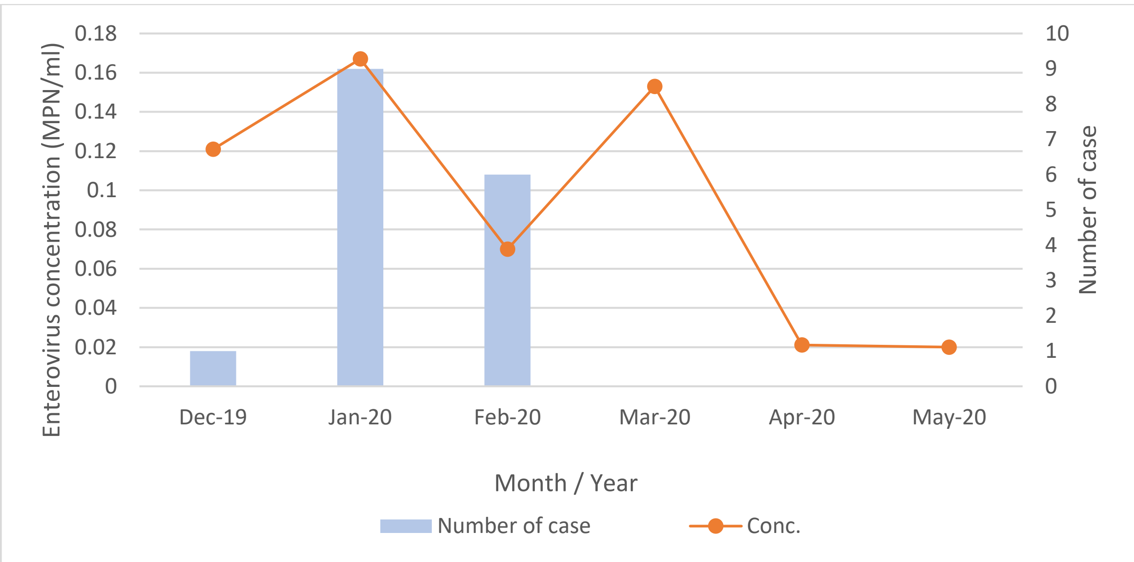


Figure 7. Trend of number of case in month and enterovirus concentration

CHAPTER V

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In this study, we found that enterovirus concentrations in wastewater from each wastewater treatment plant was not correlated. In each wastewater treatment plants they has different enterovirus concentration and prevalence. Moreover, microbial indicator cannot used as the surrogate for enterovirus in the wastewater. Thus, direct monitoring of enterovirus in wastewater is still necessary. The correlation between clinical data of enterovirus infection case and hand, foot, and mouth disease and the prevalence of enterovirus in the environment were also studied and the result showed there were not correlation.

Based on seasonality pattern data of enterovirus during 6 months in Thailand, it showed that they were no correlation between temperature and humidity and the prevalence of enterovirus in wastewater and they were no specific season trend of enterovirus in Thailand. However, this can be the new information that the prevalence of enterovirus in wastewater was various and it needs to take more attention to the factor that affects the prevalence of enterovirus in the wastewater. Thus, environmental surveillance is crucial.

5.2 Recommendation

We recommended monitoring the prevalence of enterovirus in the wastewater treatment plant because from this study the number of enterovirus detection from each plant is low that be an obstacle for statistical analysis. Furthermore, environmental surveillance is crucial to make more understanding of enterovirus in environment and wastewater treatment plant.

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