Thesis progression

Title: Mapping the viral etiology detected by NGS of acute febrile illness patients through a systematic review and meta-analysis system

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1. Background and rationale

Acute febrile illness (AFI), also known as acute unexplained illness (AUI), is a kind of fever sickness that occurs suddenly and without warning. It is common in tropical and sub-tropical areas, of which Thailand is one of them. AFI can be caused by infection of a wide variety of pathogens (viruses, bacteria, or protozoa) or non-infection (such as autoimmune diseases, transfusion reactions, and drug hypersensitivity). Usually, such fevers resolve without treatment, but fevers may result in severe and potentially fatal illnesses, which are treatable if caught early and treated properly. Clinical characteristics of AFI are similar to other fever disorders, such as a high temperature of more than 37.5 ℃, a headache, arthralgia, and myalgia. In the current studies in Southeast Asia, viruses (33.0%), bacteria (20.6%), protozoa (2.4%), and co-infections (3.4%) were shown to be the main causes of AFI [1]. Viruses are a major cause, as previously stated. The most common cause of AFI has been identified as the dengue virus, followed by influenza virus and Chikungunya virus. Also found that unknown/others (40.6%) had a lack of method diagnosis and laboratory confirmation. During the previous 21 years (January 1998–March 2019), fatalities with AFI in the Southeast Asia area occurred at a rate of 0.5%.

The majority of methods was serology test with traditional diagnosis, in which the data might not be comprehensive. Currently, the most recognized cause of diseases has gradually shifted to other factors. However, available diagnostic tools have a limited ability to detect the virus that causes the emerging disease as well as new or unexpected virus strains that may be the cause of the disease. There has been the re-emergence of viruses that were previously under control or novel viruses that have never been identified in clinical settings. This may lead to misdiagnosis, ineffective treatment, and disease spreading, leading to a worldwide pandemic and a public health issue. Thus, surveillance and detection of disease-causing viruses are therefore essential.

Metagenomic next-generation sequencing (mNGS) is a platform that can simultaneously identify genetic material (genomes) from entirely different kingdoms of organisms. It is, a massively parallel sequencing technology that provides ultra-high throughput, scalability, and speed. The method has the ability to identify

anomalies across the genome, including substitutions, deletions, insertions, duplications, copy number alterations (gene and exon) and chromosomal, by determining the order of nucleotides in complete genomes or specific sections of DNA or RNA. NGS enables laboratories to swiftly sequence complete genomes, deeply sequence specific sections, research the human microbiome, and discover new pathogens.

In this study, we performed a systematic review and meta-analysis of the data from databases to investigate the virome detected by mNGS in AFI and determine the association between virome and AFI. Moreover, the phylogeny of candidate viruses in AFI patients will be evaluated by using phylogenetic analysis. The virome profile and phylogenetic of candidate viruses obtained from this study will provide the baseline information for studies of AFI, which can be applied in the development of standard methods, and effective treatment of AFI in the future to decrease misdiagnosis of unknown AFI.

2. Objectives

2.1 To identify viruses detected by mNGS in AFI patients by using meta-analysis

2.2 To determine the prevalence of candidate virus AFI patients

2.3 To determine the phylogenetic of the candidate virus AFI patients in north-eastern Thailand

3. Hypothesis/ Research question

There is unexpected virus circulating in acute febrile illness patients which can be detected by metagenomic next-generation sequencing (mNGS).

4. Conceptual framework

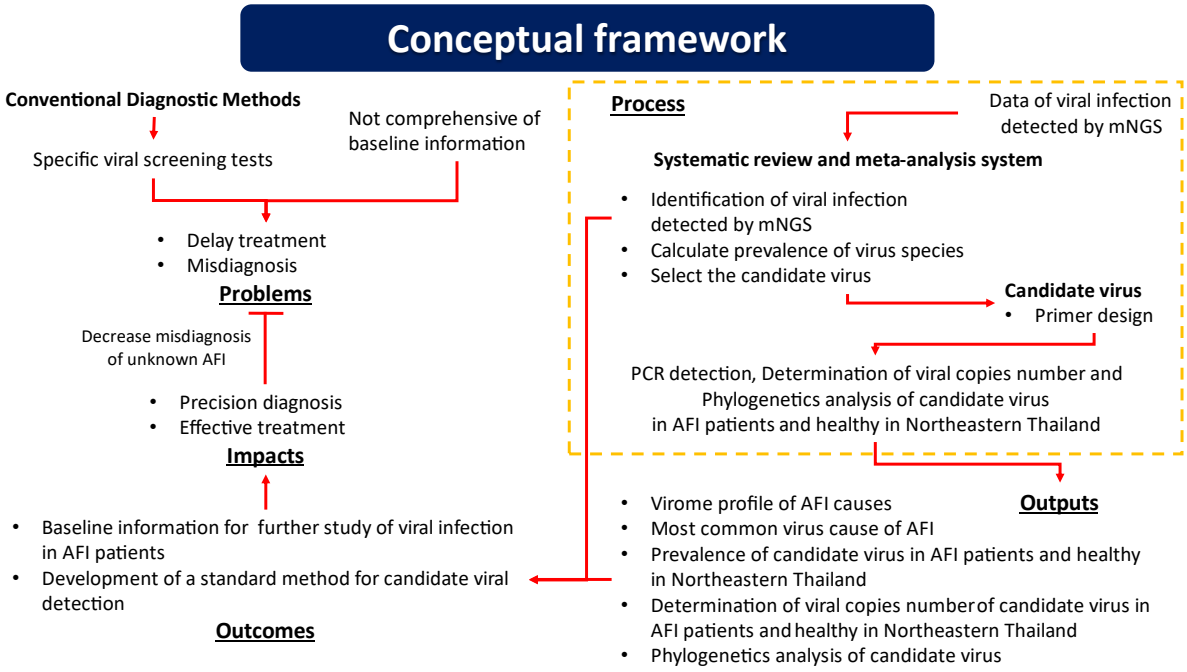

Figure 1 Conceptual framework

Acute febrile illness (AFI) is a kind of fever sickness that occurs suddenly and without warning and is caused by viruses, bacteria, and parasite infections. Diagnostic methods of AFI that conventional diagnosis methods for specific viral screening tests, which are not comprehensive of baseline information, lead to misdiagnosis, ineffective treatment, and the spreading of disease that may lead to a worldwide pandemic and a public health issue.

In this study, we review independently conducted a systematic search for observational studies to investigate the viruses, both known and newly discovered by mNGS in AFI patients and determine the association between virome and AFI. The systematic literature review and meta-analysis were undertaken according to PRISMA guidelines. to analyze the odds ratios or prevalence with 95% confidence intervals (CIs) and p-values in random-effects models due to the heterogeneity of study populations. Statistical heterogeneity was explored using Cochrane's Q Test (χ 2) and the I2 statistic, which indicates the proportion of variance of the summary effect attributable to between-study heterogeneity. A $p < 0.10$ was considered statistically significant heterogeneity, while $I2 \leq 25\%$ and $\geq 75\%$ were deemed low and high heterogeneity, respectively. Sensitivity and influence analyses were conducted based on the study size. Data analysis was completed in Review Manager. To assess publication bias, we analyze the funnel plot. And also, selecting the candidate virus for primers designed to be detected by PCR in AFI patients in north-eastern Thailand. In addition, the

determination of viral copies number and phylogenetic analysis will be used to investigate the phylogeny of the candidate viruses.

The virome profile of AFI causes, most common virus cause of AFI, prevalence, Viral copies number and phylogenetic analysis of candidate and healthy viruses obtained from this study will provide baseline information for studies of AFI, which can be applied in the development of standard methods and effective treatment of AFI in the future to decrease misdiagnosis of unknown AFI.

5. Study design

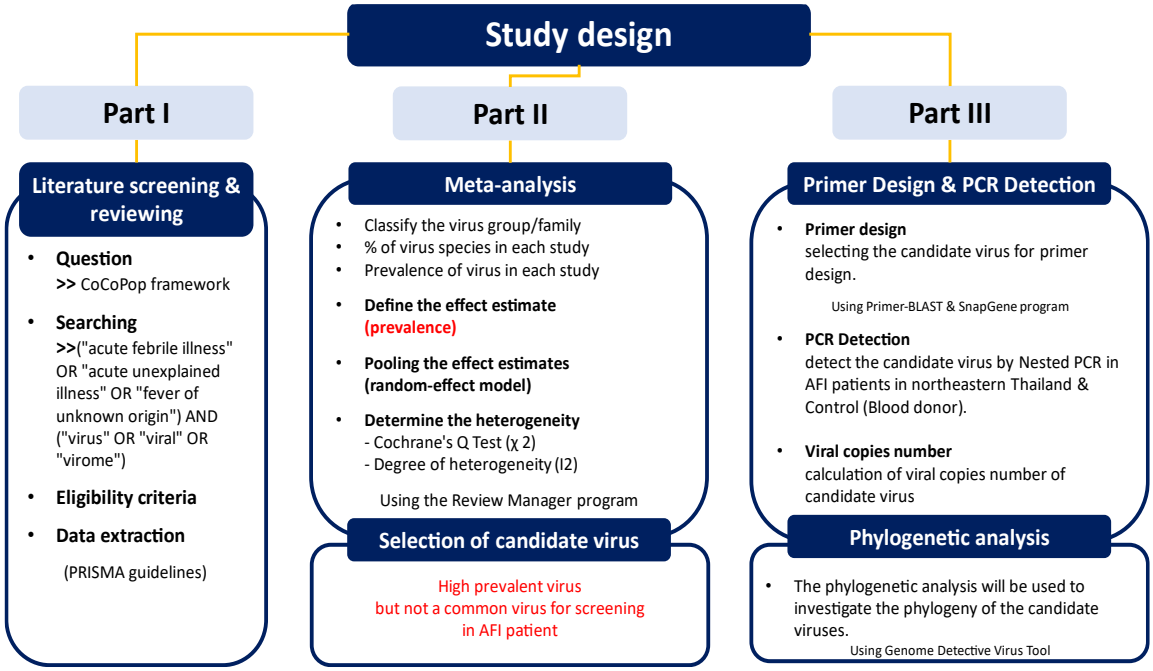

Figure 2 Study design

Part I: Literature Screening and Reviewing: The systematic literature review and meta-analysis were undertaken according to PRISMA guidelines.

Part II: Meta-analysis, to analyze the odds ratios or prevalence with 95% confidence intervals (CIs) and p-values in random-effects models due to the heterogeneity of study populations. Statistical heterogeneity was explored using Cochrane's Q Test (χ 2) and the I2 statistic, which indicates the proportion of variance of the summary effect attributable to between-study heterogeneity. A $p < 0.10$ was considered statistically significant heterogeneity, while $I2 \leq 25\%$ and $\geq 75\%$ were deemed low and high heterogeneity, respectively. Sensitivity and influence analyses were conducted based on the study size. Data analysis was completed in Review Manager. Selection of candidate virus: selecting the candidate virus that is the critical cause of AFI.

Part III: Primer Design and PCR Detection, Design primers for the candidate virus to be detected in AFI patients in north-eastern Thailand by PCR and calculate the viral copies number. Phylogenetic analysis: a phylogenetic analysis will be used to investigate the phylogeny of the candidate viruses.

6. Thesis progression

- **Previous progression**

We selected the candidate virus with the highest prevalence from the meta-analysis, and the results found that the first three with the highest prevalence are the torquevirus in the Anelloviridae family, around 20%. Also, this virus is not a common one for routine screening tests of AFI (**Figure 3**).

So, we were focused on these viruses for primer design and PCR detection.

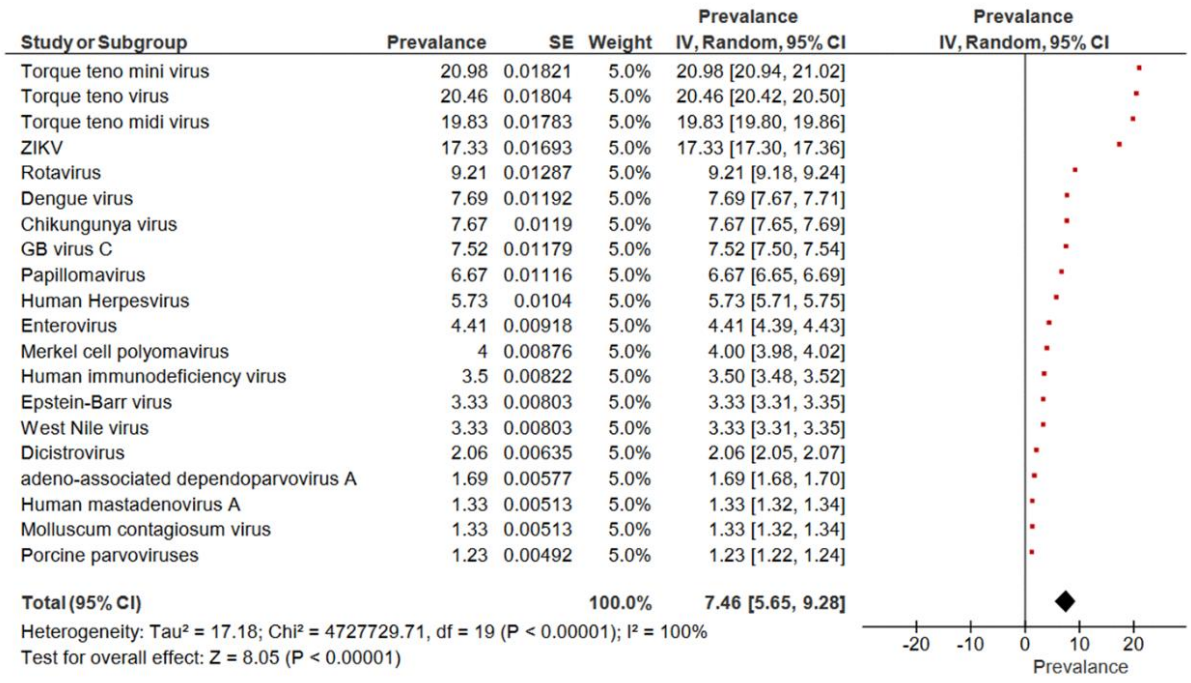

Figure 3 Prevalence of common viruses

- **Progression report**
  - **Primer design**

We review the data about the PCR primer that was used to detect the Torquevirus in humans. So, we found the primer set that was used for PCR detection in Table 1 (Ninomiya et al.). And then analyze the properties of the primer in the next step.

TABLE 1. Positions and nucleotide sequences of oligonucleotide primers used for amplification of TTV, TTMDV, and TTMV

Primer	PCR round	Polarity	Nucleotide position	Nucleotide sequence (5′ to 3′)[a]
Universal				
NG779	First	Sense	99-118[b]	ACWKMCGAATGGCTGAGTTT
NG780	First	Sense	99-118[b]	RGTGRCGAATGGYWGAGTTT
NG781	First	Antisense	208-227[b]	CCCKWGCCCGARTTGCCCCT
NG782	First	Antisense	208-227[b]	AYCTWGCCCGAATTGCCCCT
TTV-specific				
NG785	Second	Antisense	192-212[b]	CCCCTTGACTBCGGTGTGTAA
TTMDV-specific				
NG795	Second	Sense	78-97[c]	SGABCGAGCGCAGCGAGGAG
NG796	Second	Antisense	146-165[c]	GCCCGARTTGCCCCTAGACC
TTMV-specific				
NG792	Second	Sense	195-214[d]	TTTATGCYGCYAGACGRAGA
NG793	Second	Sense	195-214[d]	TTTAYCMYGCCAGACGGAGA
NG794	Second	Sense	195-214[d]	TTTATGCCGCCAGACGRAGG
NG791	Second	Antisense	247-266[d]	CTCACCTYSGGCWCCCGCCC

[a] W denotes A or T, K denotes G or T, M denotes A or C, R denotes A or G, Y denotes C or T, S denotes C or G, and B denotes C, G, or T.

[b] Nucleotides were numbered in accordance with the prototype TTV isolate of TA278 (AB017610).

[c] Nucleotides were numbered in accordance with the prototype TTMDV isolate of MD1-073 (AB290918).

[d] Nucleotides were numbered in accordance with the prototype TTMV isolate of CBD231 (AB026930).

- **Primer check**

In this step, we analyze the primer properties by using the Oligo analyzer program. The results show that the properties of this primer set are good and according to the primer guidelines. The G/C content should be between 35 and 80%, with a melting temperature (Tm) of 50 and 60 °C. Primer pairs should have a Tm

within 5°C of each other. Primer pairs should not have complementary regions such as non-homo-dimer, non-hetero-dimer, and non-hairpin loops.

- **Primer alignment**

We aligned the primer by using the NCBL primer blast. The result found that this primer set can bind with only the Anelloviridae family and show different PCR product sizes for Torquevirus and other virus species in this family. So, it is appropriate to be used for this study.

Also, we retrieved the data for the NCBI virus sequence of three genera of Torquevirus as 490 sequences. To determine whether the specific primers can bind to the newly uploaded sequence or not, Using the SnapGene program. The results show that this universal primer set can bind with 272 sequences from three TTV genera, and 218 sequences are another region the primer cannot bind.

- **Prediction of PCR product**

We randomly selected 15 sequences from 490 sequences of three genera of Torquevirus on the NCBI virus sequence for PCR detection by in silico methods using SnapGene. The results show that this primer can detect all sequences with a PCR product size of around 137 bp (Figure 4).

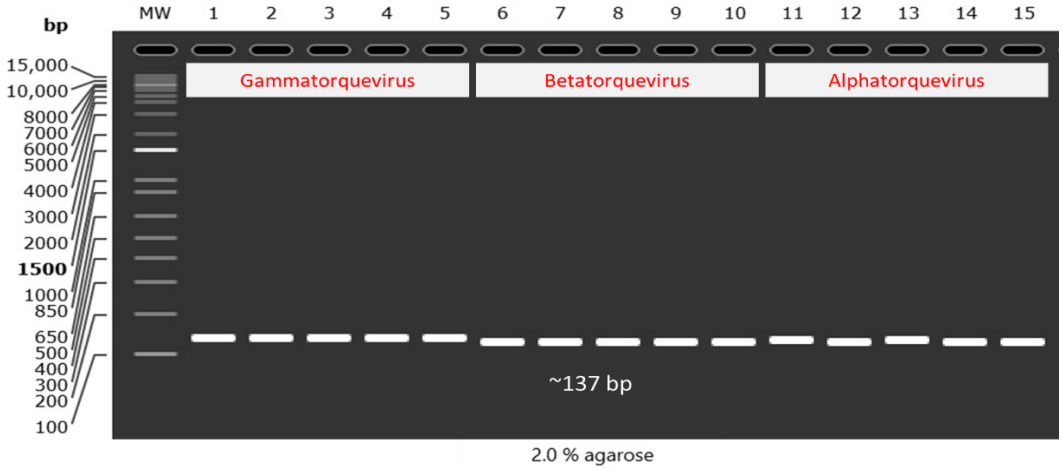

Figure 4 Gel electrophoresis of PCR product of Torquevirus detection prediction

- **Further Works**

- Collection of healthy control blood from blood bank
- Nucleic acid extraction of control samples
- Primer test
- PCR detection of TTV in AFI cases & healthy control
- Determination of viral copies number of TTV
- Phylogenetic analysis of TTV

7. Research plan

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