MOLECULAR EPIDEMIOLOGY AND EVOLUTIONARY STUDIES OF HUMAN RHINOVIRUS AND ENTEROVIRUS 68 IN THAILAND

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การศึกษาระบาดวิทยาและวิวัฒนาการของเชื้อไรโนไวรัส และเอนเทอโรไวรัส 68 ในประเทศไทย

นางสาวปิยดา หลินศุวนนท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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ปิยดา หลินศุวนนท์: การศึกษาระบาดวิทยาและวิวัฒนาการของเชื้อไรโนไวรัส และเอนเทอโรไวรัส 68 ในประเทศไทย (MOLECULAR EPIDEMIOLOGY AND EVOLUTIONARY STUDIES OF HUMAN RHINOVIRUS AND ENTEROVIRUS 68 IN THAILAND.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นพ.ยง ภู่วรวรรณ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.สัญชัย พยุงภร, 125 หน้า.

โรคติดเชื้อในระบบทางเดินหายใจ คือ โรคที่เกิดขึ้นกับอวัยวะในบระบบทางเดินหายใจทั้งส่วนบนและส่วนล่าง สาเหตุของโรคที่พบบ่อย คือ ติดเชื้อไวรัสซึ่งมีอยู่มากกว่า 200 สายพันธุ์ หนึ่งในไวรัสที่มีบทบาทสำคัญต่อการก่อโรคติดเชื้อในระบบทางเดินหายใจ คือ เชื้อไรโนไวรัสและเอนเทอโรไวรัส ซึ่งมีความหลากหลายทางสายพันธุ์มาก ทำให้มีโอกาสติดเชื้อไวรัสนี้ได้บ่อยครั้ง ในปี 2007 ได้มีรายงานการค้นพบเชื้อไรไนไวรัสสายพันธุ์ใหม่ หรือ สปีชีส์ ซี ซึ่งพบว่าการติดเชื้อไวรัสสายพันธุ์นี้ มีแนวโน้มที่โรคจะมีความรุนแรงหรือเป็นโรคเรื้อรังมากกว่าการติดเชื้อไรโนไวรัสสายพันธุ์อื่นๆ ทำให้เชื้อไรโนไวรัส สีปีชีส์ใหม่นี้อยู่ในความสนใจของผู้วิจัยจำนวนมาก แต่อย่างไรก็ตามเนื่องจากเชื้อไรโนไวรัส สปีชีส์ ซี ไม่สามารถจะเพาะเลี้ยงในเซลล์ได้ จึงทำให้ข้อมูลด้านระบาดวิทยา อาการทางคลินิค ความสัมพันธ์เชิงชีวโมเลกุลของแต่ละสายพันธุ์ และวิวัฒนาการของไวรัสสายพันธุ์นี้มีอยู่อย่างจำกัด นอกจากนี้ในช่วงระยะเวลาใกล้เคียงกันก็ได้มีรายงานการอุบัติซ้ำของโรคติดเชื้อในระบบทางเดินหายใจซึ่งมีสาเหตุจากเชื้อเอนเทอโรไวรัส ซีโรไทป์ 68 การศึกษาวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาระบาดวิทยาของเชื้อไรโนไวรัส และเอนเทอโรไวรัส โดยเฉพาะเชื้อเอนเทอโรไวรัส 68 โดยทำการตรวจวิเคราะห์ตัวอย่างสารคัดหลั่งซึ่งเก็บได้จากกลุ่มผู้ป่วยโรคติดเชื้อในระบบทางเดินหายใจเด็กอายุต่ำกว่า 15 ปี จากโรงพยาบาลต่างๆ ในกรุงเทพมหานคร จำนวนทั้งสิ้น 2,083 ตัวอย่างโดยทำการตรวจวิเคราะห์เชื้อไรโนไวรัส เอนเทอโรไวรัส อินฟลูเอนซาไวรัส และเชื้อไวรัสก่อโรคทางเดินหายใจอื่นๆ ด้วยวิธี polymerase chain reaction ผลการวิจัยพบว่าร้อยละ 45 ของประชากรศึกษาตรวจพบเชื้อไวรัสอย่างน้อย 1 สายพันธุ์ และตรวจพบเชื้อไรโนไวรัสร้อยละ 13.8 โดยเชื้อไรโนไวรัส สปีชีส์ซีเป็นสายพพันุ์ที่พบมากที่สุดคิดเป็นร้อยละ 48 เชื้อเอนเทอโรไวรัส 68 พบเพียงร้อยละ 1.4 ของประชากรศึกษาทั้งหมด เชื้อไรโนไวรัสพบได้มากในกลุ่มเด็ก โดยเฉพาะเด็กที่อายุต่ำกว่า 5 ปี ในขณะที่เชื้อเอนเทอโรไวรัส 68 พบมากในเด็กโต ความสัมพันธ์ของความรุนแรงของโรคต่อสายพันธุ์ไวรัสนั้น พบว่าเชื้อไรโนไวรัส สปีชีส์ ซีและเชื้อเอนเทอโรไวรัส 68 มีแนวโน้มที่จะสัมพันธ์กับโรคในระบบทางเดินหายใจส่วนล่างมากกว่าเชื้อสายพันธุ์อื่นๆ ผลการวิเคราะห์ความสัมพันธ์ของค่าพารามิเตอร์ต่างๆ และอัตราเชื้อไวรัสที่ตรวจพบนั้น สรุปได้ว่า การระบาดของเชื้อไวรัสในประเทศไทยนั้นไม่มีฤดูกาลหรือแบบแผนที่จำเพาะ เป็นลักษณะที่สัมพันธ์กับค่าความชื้นสัมพัทธ์ในอากาศทำให้พบอุบัติการณ์ของโรคมากในฤดูฝน และสามารถตรวจพบได้ตลอดทั้งปีเนื่องมาจากลักษณะอากาศร้อนชื้นของประเทศ ผลการวิเคราะห์ข้อมูลวิวัฒนาการของเชื้อไรโนไวรัสพบว่า เชื้อไรโนไวรัส สีปีชีส์ ซีนั้นมีบรรพบุรุษร่วมกับ สีปีชีส์ เอ และเริ่มแยกออกจากกันเมื่อประมาณ 750 ปีก่อน และแพร่กระจายในประชากรทั่วโลกเป็นเวลาอย่างน้อย 10 ปี อีกทั้งวิวัฒนาการของเชื้อไวรัสนั้นเกิดขึ้นภายใต้สภาวะ purifying selection

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5375364530 : MAJOR BIOMEDICEAL SCIENCE
KEYWORDS : EPIDEMIOLOGY/ RHINOVIRUS/ ENTEROVIRUS 68/ EVOLUTIONARY HISTORY

PIYADA LINSUWANON: MOLECULAR EPIDEMIOLOGY AND EVOLUTIONARY STUDIES OF HUMAN RHINOVIRUS AND ENTEROVIRUS 68 IN THAILAND. ADVISOR: PROF. YONG POOVORAWAN, MD., CO-ADVISOR: ASST. PROF. SUNCHAI PAYUNGPORN, Ph.D.,125 pp.

Acute respiratory tract illness is respiratory diseases that affect the air passages frequently occurs in rainy and winter seasons causing many diseases such as common cold, pneumonia, bronchitis, and asthma exacerbation. Many kinds of virus have been well recognized associated with the respiratory diseases. Publications worldwide have reported on the emerging of human rhinovirus (HRV) species C in 2007 and the re-occurrence of human enterovirus 68 (HEV68), a rarely detected pathogen usually causes respiratory illness. At present, epidemiological data, clinical complication and evolutionary history regarding these viruses in particular on the Asia continent are still limited. To address these concerns, this study aimed to establish comprehensive population-based surveillances during 2006-2012 (n = 2083), and provided evidence for their impacts in childhood respiratory tract illnesses. Employing PCR approaches for common respiratory virus detection, our study revealed that ~45% of enrollment children were infected with at least one viral agent, and HRV being one of the most common viruses detected with annual prevalence of 13.8%. Results displayed that infections by HRV in both of influenza-like illnesses and lower respiratory tract illness patients predominantly targeted very young children with viral pneumonia, bronchiolitis and wheezing were the common discharged summary. The majority of HRV species identified in Thailand was HRV-C (48%). In contrast to HRV, HEV68 prevalence was estimated at 1.4% with main target group in older children. No significant respiratory complication was found between number of case of HRV, HEV68 and other respiratory viruses. The activity of respiratory viruses identified in Thailand showed significantly direct correlation with the relative humidity. Taken all evidence together, this study suggested that respiratory viruses circulating in Thailand were widely diverse, and the epidemics profile displays species, season, and year variations as the consequence of less distinct among seasonal oscillation. Furthermore, results obtained from Bayesian MCMC analysis suggested that HRV-C was not a recently emerged human pathogen. Otherwise, it was probable the descendent of the closely related HRV-A for >750 yrs, and at least presented in human community for 10 yrs. Analysis of evolutionary force sharped HRV and HEV68 diversification indicated, as similar to other picornavirus, the evolutionary status of these viruses were relatively stable and undergo with strong purifying selection.

Field of Study : Medical Sciences Student's Signature .....

Academic Year : 2012 Advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBERVIATIONS

HRV	=	Human Rhinovirus
HEV	=	Human Enterovirus
RSV	=	Respiratory syncytial virus
HMPV	=	Human metapneumovirus
PV	=	Poliovirus
CV	=	Coxsackie virus
Echo	=	Echovirus
ARTI	=	Acute respiratory tract infection
ILI	=	Influenza-like illness
URTI	=	Upper respiratory tract illness
LRTI	=	Lower respiratory tract illness
UTR	=	Untranslated region
VP	=	Viral capsid protein
RNA	=	Ribonucleic acid
NP	=	Nasopharyngeal aspiration
NS	=	Nasal swab
DNA	=	Deoxyribonucleic acid
+ssRNA	=	Positive single-stranded RNA
-ssRNA	=	Negative single-stranded RNA
5′CL	=	5′cloverleaf-like motif
IRES	=	Internal ribosome entry site
ORF	=	Open reading frame
poly-A	=	Poly-adenine tract
Cre	=	*Cis*-replicating element
RdRp	=	RNA-dependent RNA polymerase
NIm	=	Neutralizing immunogenic site
ICAM-1	=	Intercellular adhesion molecule-1
LDLR	=	Very low-density lipoprotein receptor
SCARB2	=	Human scavenger receptor class B2

PSGL1 = P-selectin glycoprotein ligand-1

DEF = Decay-accelerating factor

IL = Interleukine

IP-10 = CXCL10/interferon-inducible protein 10

CNS = Central nervous system

PCR = Polymerase chain reaction

RT-PCR = Reverse transcription polymerase chain reaction

MCMC = Markov chain Monte Carlo

ω = Non-synonymous substitution to synonymous substitution ratio (*dN/dS* ratio)

yrs = Years

bp = Base pair

nt = Nucleotide

NJ = Neighbor-joining

SLAC = Single likelihood ancestor counting model

FEL = Fixed-effects likelihood model

PCM = Phylogenetic compatibility matrix

TMRCA = The most common recent ancestor

NS = Nonsynonymous amino acid

NC = Non-conservative amino acid

NC-NS = Non-conservative variation

C-NS = Conservative nonsynonymous variation

s/s/y = Substitution per site per year

r_s = Pearson correlation coefficient

p = Probability value

pp = Posterior probability

HPD = Highest posterior density

Temp = Temperature (degree Celsius)

RH = Relative humidity

CHAPTER I

GENERAL INTRODUCTION

Background and rationale

According to the WHO respiratory disease definition, acute respiratory tract illnesses (ARTIs) are respiratory diseases that affect the air passages including the nasal cavities, pharynx, larynx, trachea, bronchi and lungs. ARTI is a term used to describe the diseases range from acute infections, such as pneumonia, wheezing illness and bronchitis, to acute exacerbations in chronic conditions e.g., asthma and chronic obstructive pulmonary disease. All of these diseases are major causes of morbidity in all ages, particularly during the first year of life and among elder persons, which lead to the disease burden in public health worldwide. Although viruses and bacteria (*Streptococcus pneumoniae* and *Haemophilus influenza*) have been recognized as causative organisms of ARTIs, the most frequent causes of ARTIs are originated from viruses. Many studies have reported that several respiratory viruses influence ARTIsI including influenza virus (Flu), human metapneumovirus (HMPV), parainfluenza virus (Paraflu), respiratory syncytial virus (RSV), Human adonovirus (AdeV), and human coronavirus. Of the overall percentage of the illnesses, human rhinovirus (HRV) is one of the most common causative agents identified which accounts for 25-50% of all infections [1, 2].

Exposure to HRV strains begins early and continues throughout life. The frequency of HRV infection is generally high among children below 5 years of ages and elders. HRV infections in immunocompetent patients represent only as bystander viruses, or cause subclinical consequences such as common cold. HRV has significantly become a targeted respiratory virus due to its ability to replicate in the lower respiratory tract, and its contribution to the development of extensive ranges of respiratory disorders including pneumonia, viral bronchiolitis, bronchitis, and exacerbation in patients with chronic respiratory diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis [3, 4]. Accumulating evidence has shown that HRV infections lead to idiopathic pulmonary fibrosis, fatal pneumonia, and respiratory failure in immunocompromised patients [5-8]. Human Enterovirus (HEV) such as coxsackie virus (CV) infection can also cause respiratory tract illness and gastroenteritis with high grade fever. Infection by AdeV is associated with bronchitis and acute conjunctivitis. Nonetheless, co-infections by several

pathogens occur frequently among ARTIs. Therefore, it is not possible to differentiate between virus infections and their clinical consequences based on clinical signs or radiology. Further investigations are needed to ascertain the role of HRV and other viruses exacerbating factors for ARTIs. Epidemiologic surveillances of respiratory virus infection in many areas have been identified to demonstrate two relevant distribution patterns of HRV. In some areas, the specific serotypes were more frequently identified and tended to persist [9, 10]. As a consequence of the antigenic drift, clear increase overtime in the proportion of HRV isolates representing higher number of serotypes or unprecedented serotypes were found.

Currently, knowledge in HRV epidemiology, pathogenicity, and disease association are sufficient for developing of the treatment and prevention modalities. In recent years, by using highly sensitive molecular techniques, novel viruses and some of sporadically detected viruses have increasingly been identified and investigated. Among these, human enterovirus serotype 68 (HEV68) has been recognized as a re-emerging respiratory virus detected in many countries worldwide with high detection incidence in 2008. HEV68 shares basic characteristics with HRV and is genetically and antigenically similar to HRV serotype 87 (HRV87). HEV68, so far, has been becoming an increasingly important etiological agent as it is the only pathogen identified in respiratory specimens obtained from fatal cases reported in the retrospective studies of the Philippines and Japan [11-13]. Therefore, awareness of its global distribution, cohort epidemiologic studies and pathogenic role of this virus should be concerned.

Taken all these together, this study aimed to characterize of epidemiological profile, genetic variability, and clinical presentation of HRV and HEV68 in Thailand during 6 different epidemic seasons from February 2006 to August 2012 and achieve the evolutionary history of these world-wide distributed viruses. This study plan to achieve the overall objective by pursuing these studies as follows;

Part 1: Molecular epidemiological studies of HRV and HEV68 among pediatric patients with acute respiratory tract illnesses in Bangkok, Thailand.

Part 2: Evolutionary dynamics of HRV and HEV68

CHAPTER II

REVIEW OF RELATED LITERATURES

General characteristics of HRV and HEV

The genus *Enterovirus* in the family *Picornaviridae* is a large group of economically and socially very important human viruses comprising human rhinovirus (HRV) and enterovirus (HEV) species. HRV is one of the most common etiological agents of ARTIs. It was originally isolated in United State in the 1950s from patients with common cold. To date, more than 100 immunologically distinct serotypes have been identified. Infections by this virus in healthy individuals are implicated in approximately (~) 50% of asymptomatic or self-limited upper RTIs with a short incubation period of 1 to 3 days. In contrast, members within the enterovirus species are well recognized as causative agents that are associated with a spectrum of diseases, ranging from subclinical ARTIs to more severe manifestations of severe neurological disorders and gastrointestinal illnesses, such as encephalitis, meningitis, acute flaccid paralysis, pancreatitis [14], myocarditis [15], acute haemorrhagic conjunctivitis, hand-foot-mouth, multisystem haemorrhagic disease of newborns, myalgia, and neonatal sepsis-like diseases [16-19]. Both of HRV and HEV share an identical genomic organization and gene functions. They also have similar functional RNA secondary structures which result in reclassification of the genus *Rhinovirus* as they are now members within the genus *Enterovirus* (International Committee on Taxonomy of Viruses, 2011). In general, they are small non-enveloped viruses, spherical structure of diameter of 25 to 30 nm. They possess a positive single-stranded RNA genome (+ssRNA) of ~7.1 to 7.2 kb in length for HRV and 7.4 to 7.5 for HEV, which are encased by a highly icosahedral capsid.

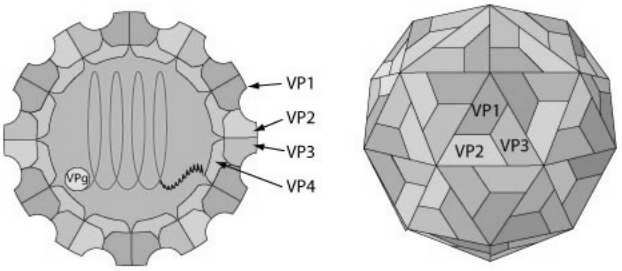

Figure 1: Schematic view of picornavirus virion [20].

The viral genome is covalently linked to virus-encoded peptide VPg protein at the 5'-uridine of the genome. HRV and HEV virion have relatively simple structures. The compositions of HRV and HEV virion are described as follows;

1. Capsid protein

Viral capsid protein (VP) serves multiple functions in many stages of the virus life cycle such as protecting the viral RNA genome from degradation by environmental RNAses, determining host and tissue tropism by recognition of cell-specific receptors, penetrating target cells, and delivering the viral RNA into the host cell [21]. The capsid structure of these viruses is composed of 60 subunits of each 4 structural proteins-VP1 to VP4 arranged on a T = 1 icosahedral lattice. The fundamental capsid architecture is the same in all picornavirus family's members. The outer surface of the capsid is formed of parts of VP1, VP2 and VP3 proteins which are in the range of 240 to 290 residues. VP1 are located at the five-fold symmetry axes, and VP2 and VP3 alternating around the three-fold symmetry axes. The internal capsid is composed the N-termini of VP1 to VP3 and all 60 VP4 molecules. The N-terminal residue of VP4 is covalently bonded to a myristic acid, giving the capsid five symmetry related myristoyl moieties around the inner surface of the icosahedral fivefold axis. The thickness of the capsid shell is various but averages around 5 nm. Each capsid protein taking the form of 8 stranded antiparallel β-sheet structures, β-barrel/βB-BC-βC-CD-βD-DE-βE-EF-βF-FG-βG-GH-βH-HI-βI/β-barrel, with jelly roll topology that is common to many viral capsid protein (Fig 2). The mature capsid proteins VP1 and VP3 are exist as a convoluted set of protein sheets and loops. The loops protrude beyond the external capsid surface and play as parts of the virus antigenic sites. Furthermore, the capsid is a narrow depression around each of the fivefold axis termed the canyon, which has been recognized to be the receptor binding site for picornaviruses.

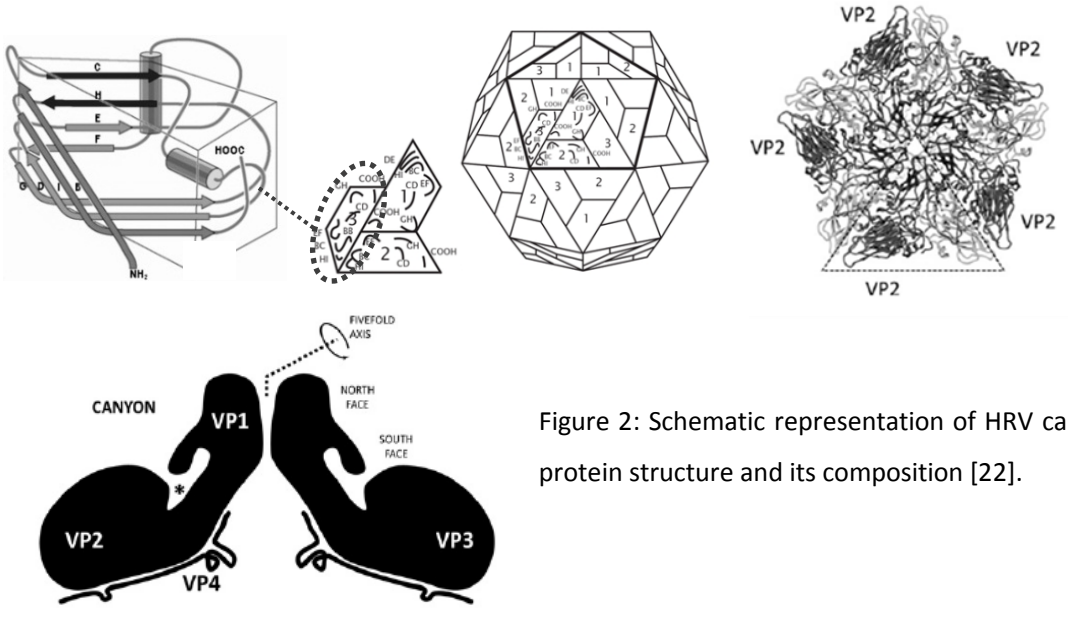

Figure 2: Schematic representation of HRV capsid protein structure and its composition [22].

Amongst all capsid proteins, VP1 is the largest and the most exposed surface capsid. VP1 is not only encompassed the pocket lined with hydrophobic residues (pocket factor), receptor binding site and accessible from a depression on the outer surface of the virus capsid, but it also comprises the majority of motifs important for interaction with neutralizing antibodies. Because VP1 protein plays the key role in picornavirus replication, therefore,many study groups try to develop antiviral drugs that target to a hydrophobic pocket in VP1. Numbers of antiviral compound such as disoxaril (WIN 51711), WIN54954, WIN61209, WIN68934, and WIN 65099 [23], which interact with the capsids of picornaviruses, have been known for a number of years. These compounds inhibit the viral attachment to the host cell by integrating, with high affinity and specificity, in the hydrophobic pocket and prevent decapsidation process, and thus abort the infection cycle. Although some of these compounds exhibited activities when administered prophylactically, some undesirable side effects have been concerned. For example, disoxaril is phase II clinical trials compound but it failed to toxicity tests [24, 25]. The third generation pleconaril (WIN 63843), which is firstly developed by Schering-Plough Corporation, is claimed to resolve the cold symptom within 1-2 days and prevent asthma exacerbations and common cold symptoms in asthmatic subjects infected by picornavirus. However, it had side effects and also some HRV strains resist to this antipicornaviral agent. Therefore, Food and Drug Administration of theUnited States, declared this drug as not approvable in phase III clinical trials [26].

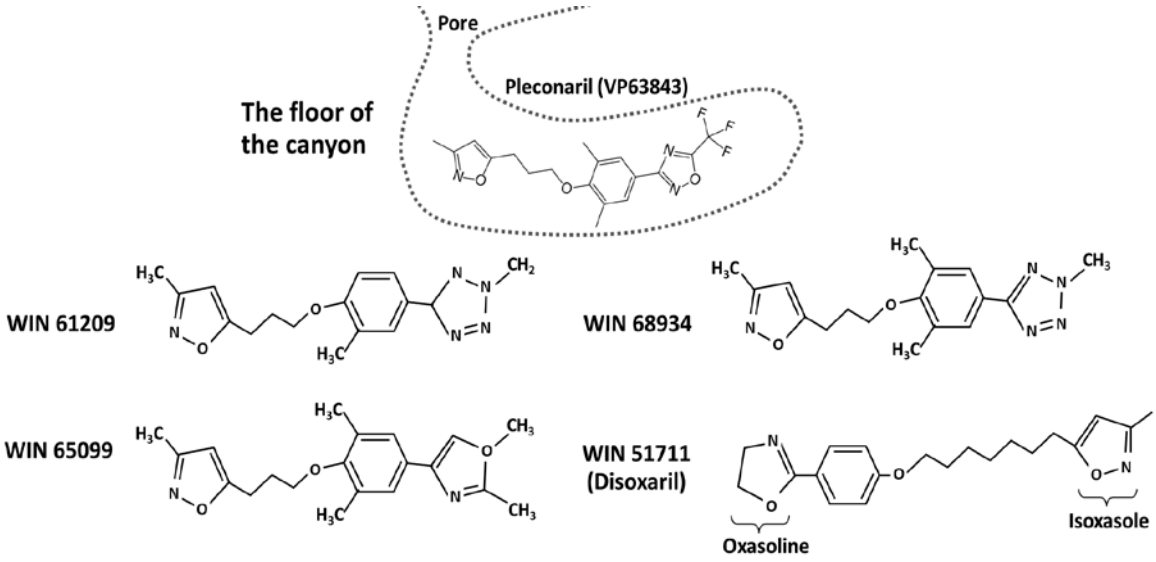

Figure 3: Example of antipicornaviral agents.

2. Viral genome

According to the limitation of viral genome size, genetic architecture of the viral genome is overlapped between structural and functional domains which have different functions that are crucial for virus survival. The viral genome is comprised of a long open reading frame (ORF) flanked by a 5′ and short 3′-untranslated region (UTR) with poly-adenine tract (poly-A) (Fig 4).

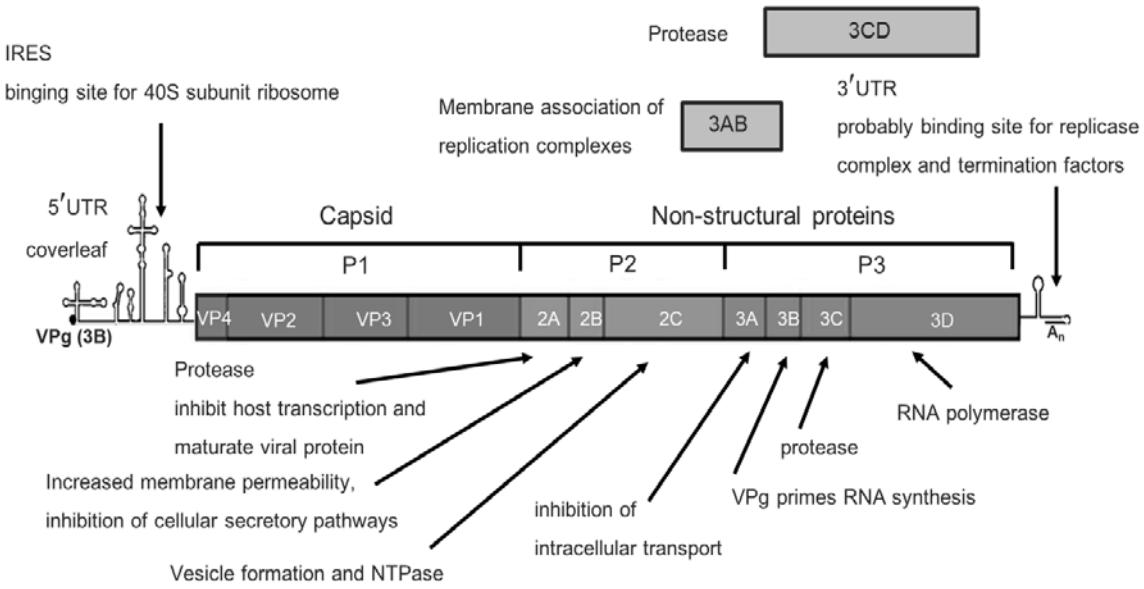

Figure 4: A Schematic representing the picornavirus genome, proteins, and their functions.

Main functions of each viral protein are combined between enterovirus and rhinovirus members. The mature proteins are shown together with the 3 main cleavage intermediates (modified from Whitton JF [27]).

The 5′UTR covers the first ~700 nt and 600 nt in length of HEV and HRV genome, respectively. This region contains highly structured secondary elements with cloverleaf-like motif (5′CL) and internal ribosome entry site (IRES). The 5′CL motif functions in the binding of viral and host cellular protein which is necessary for the initiation of RNA synthesis and also helps in converting the infectious genomes from translation to replication templates. IRES of all HRV and HEV are type 1-IRES. This structure is utilized as a binding site for 40S ribosome to mediate cap-independent translational initiation of the viral ORF. The 3′UTR begins with the ORF termination codon and extends to the genetically encoded poly-A track. The 3′UTR is thought to forms the stem loops which can be either served as a putative protein recognition motif for termination factors, promoted

replication enhancement, or can be interacted with other parts of the genome [28]. Number of studies suggested that poly-A track plays an important role in virus replication as it is served as template for VPg primer. Furthermore, synergistic interplay between the initiation factor and the poly-A tract is sufficient for the virus effective translation (Fig 5).

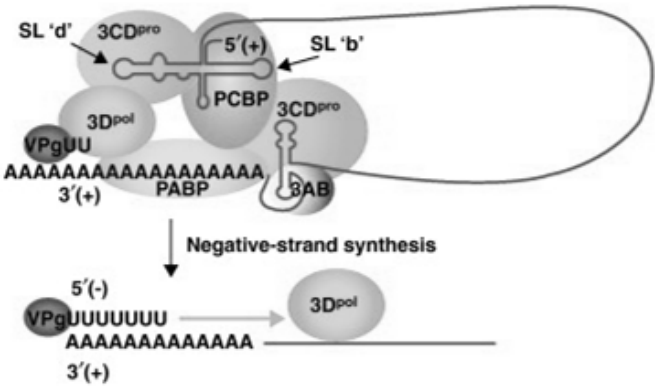

Figure 5: A model showing functions of the 5′CL and poly-A during initiation of -ssRNA synthesis.

The viral ORF can be divided into 3 main regions; structural P1 and nonstructural P2 and P3 regions. The P1 region is comprised of 4 structural genes, 1A-1D, encoding for capsid VP1 to VP4 (named in order of their sizes). VP1 is the largest capsid protein of ~40 kDa. It has been recognized as an essential part of the viral antigenic determinant, and it is also utilized as binding sites of many cellular receptors and neutralization antibodies. As for its functions, VP1 is one of the attractive targets for developing antiviral synthetic compounds. VP0 plays vital roles in the assembly of the viral capsid. It is a precursor for VP2 and VP4. The P2 and P3 regions are comprised of 7 viral genes which 2A is included into 2C, and 3A is included into 3D. The P2 and P3 encode for viral proteases ($2A^{Pro}$ and $3C^{Pro}$), helicase, membrane associated factor (2C), and RNA-dependent RNA polymerase (RdRp), which are responsible for viral replication, and protein processing. Moreover, it also functions in shutting down the host's cell protein production.

Replication cycle

Several mechanisms have been established how genome of HRV and HEV enters into the host cells. One model involves in transferring of the viral RNA into the host's cytoplasm through virus attachment at the plasma membrane and leaving the capsid at the cell surface. Other model depends on endocytosis mechanism. According to this mechanism (Fig 6), the viral genome is either released from the capsid after disruption of the endosome membrane, or only the viral RNA is released and transported across the

endosome membrane. Furthermore, the first stage of some picornaviruses infection, such as HRV, poliovirus (PV), and HEV, is mediated by the interaction of the viral capsid with specific receptors on the membrane of the susceptible cells. The binding process changes the capsid conformational in a way that transforms the virion into an altered particle which results in the loss of VP4 and the externalization of the amino terminus of VP1 follows by the release of viral RNA to cytoplasm. All stages of the virus replication cycle take place in the cytoplasm of an infected cell. The viruses must utilize host cell factors for translation, initiation, and RNA replication. In general, genome replication proceeds by replicating of parental +ssRNA into nascent -ssRNA template. The protein VPg (3B) serves as a primer for production of diuridylylated-VPg in a reaction catalyzed by RdRp. Next, the resulting molecule is transferred to the 3' end of +ssRNA or -ssRNA, and serves as primer for polymerization of full-length viral genome [29]. Like most +ssRNA viruses, the translational processes have unavoidable conflicts with the -ssRNA synthesis, since they occur in the opposing directions and also utilize +ssRNA as a template.

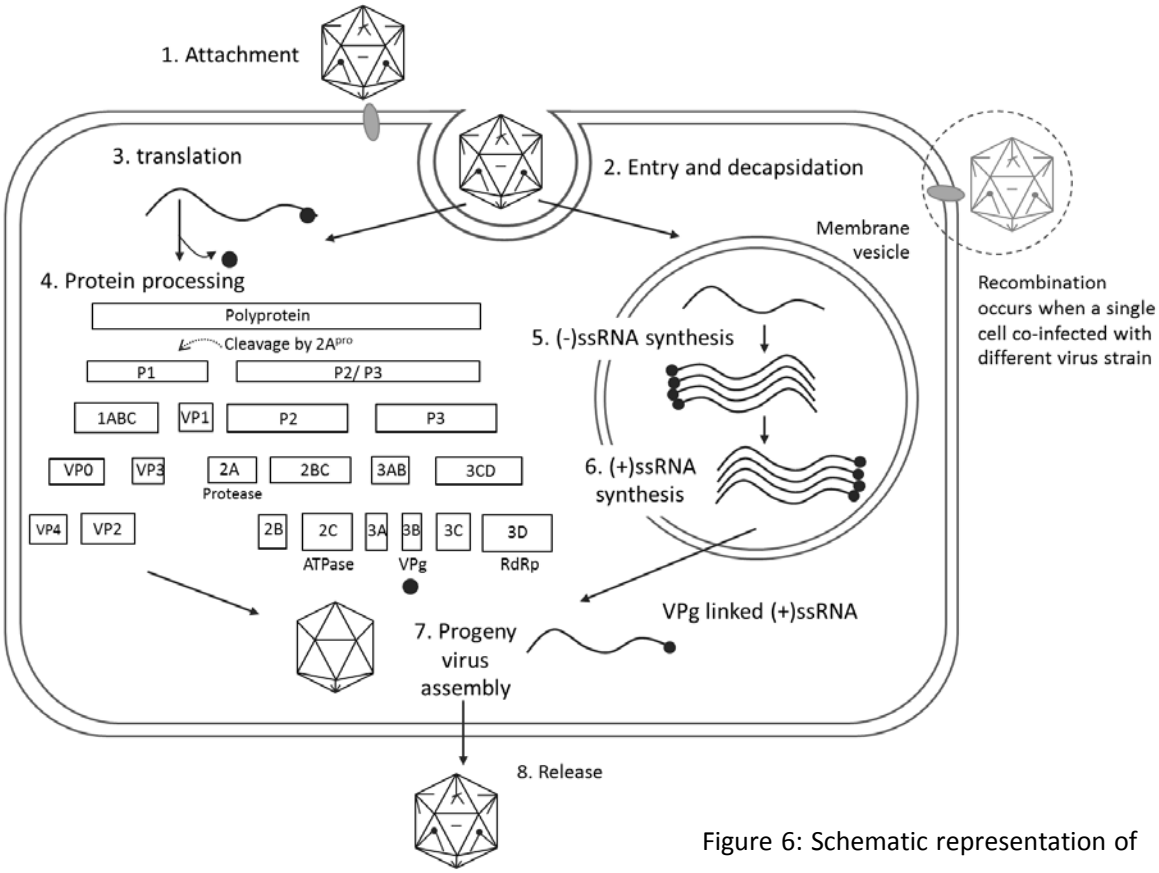

Figure 6: Schematic representation of the picornavirus replication.

As for the translational processes, the viral proteins are translated from a single ORF that is subsequently cleaved by virus encoded protease to yield precursor proteins P1, P2 and P3. P1 is a precursor for structural proteins including VP1, VP3, and VP0. It is derived from auto-catalytically cleavage of $2A^{Pro}$ at N-terminus of P1 adjacent to P2/P3 nascent polyprotein and is thereafter cleaved into a single capsid protein by $3CD^{Pro}$. Following capsid production, the majorities of nonstructural protein P2/P3 cleavages are mediated by the viral encoded $3C^{Pro}$. The structural proteins are spontaneously assembled into the icosahedral procapsid, followed by the encapsulation of the RNA genome into the provirion. The final step in viral assembly involves in the cleavage of VP0 precursor into VP2 and VP4 single proteins, resulting in mature virion. The mature virion consists of 60 copies each of 4 capsid proteins and 1 or 2 copies of VP0. In case of a cell becomes infected with the 2 different strains of HRV or HEV, recombinant viruses may be presented amongst the progenies from that cell.

Classifications of HRV and HEV

Both HRV and HEV taxonomy and classification rely on several parameters and strategies. Normally, traditional classifications rely on biological properties such as antigenic distinctions which subdivide these viruses into ‘serotypes’ (numbered sequentially). By using culture and serum neutralization strategies, HRV and HEV have been found to have tremendous genetic diversity with at least 100 immunologically distinct serotypes have been identified. However, this method is time consuming, limited to certain reference laboratories, and is also complicated by the existence of over 100 serotypes of HRV and HEV. Moreover, this method is not capable of classifying some of newly identified HRV and HEV strains. Consequently, as alternative and more sensitive methods for classification of all of HRV and HEV, current techniques have been developed based on the advantages of reverse transcription polymerase chain reaction (RT-PCR) and nucleotide sequencing of the antigenic sites that viruses utilize to evade the host’s immune system. These strategies have been commonly focused on the VP1 as this region is a major part of the antigenic sites. Some studies use VP4, VP4/VP2 and/ or a highly conserved 5’UTR for classification due to its correlation to the results from sero-neutralization assays [28, 30]. Like most of picornavirus members, HRV and HEV show substantial genetic diversities that underlie the existences of a large numbers of

antigenically distinct variants. By using molecular classification approaches, more than 100 immunological distinct serotypes of HEV can be categorized to 4 genetic clusters designed as HEV species A to D and PV. PV has several characteristic that very close to HEV species C. Separation between PV and HEV species is based on from HEV-C is based on their ability to cause poliomyelitis. For HRV, the previous defined serotypes have been classified to 2 species comprising species A and B with a total of 75 and 25 serotypes, respectively. Moreover, broader uses of molecular diagnostics in clinical specimens combined with the results from evolutionary analyses have led to the discovery of the new virus members such as HRV species C and respiratory tract disease associated HEV-104 and HEV-109 [31, 32]. The classification of new types or strains within a species is based on the similarity of nucleotides and amino acid sequences of the capsid coding regions. A new type of HRV or HEV is commonly defined by less than (<) 75% nucleotide and <85% amino acid sequence identity with known members across the VP1. HRV and HEV members in each species and clinical impact are summarized Table 1.

Even though HRV and HEV share a numbers of biological and genetic attributes, their characteristics are moderately different such as the differences in their optimal growth temperature, acid sensitivity, cell tropism, and receptor usage. HRV currently consists of 151 types. Among these 100 types are HRV-A and HRV-B and 51 designed types are HRV-C [33]. Further, 3 new types of HRV-A, HRV-A101, and HRV-A103, have also been defined on the basis of VP1 sequence divergence [34]. Despite the antigenic diversity of HRV-A and HRV-B, they attach to only 2 types of cellular receptor resulting in categorization of all of them to 2 groups including major and minor group. HRV receptor preference can be determined by the amino acid footprint variation either on the virion surface and receptor-interacting canyon region or in the carboxy-terminal VP3 or the entire VP1. The major group virus, comprising 90% of HRV (62 HRV-A and all HRV-B), uses an intercellular adhesion molecule-1 (ICAM-1) receptor to penetrate into the susceptible cells, whereas the minor group (12 HRV-A serotypes) utilizes very low-density lipoprotein receptor (LDLR) [35-38]. Furthermore, major and minor group viruses also have different antigenic site but located in the same region. For major group viruses, 4 primary neutralizing immunogenic (NIm) sites have been identified including NIm-1A (located in VP1), NIm-1B (VP1), NIm-II (VP2 and VP1) and NImIII (VP3 and VP1). For minor group virus,

3 distinct antigenic sites designed as A, B and C that is located in the same vicinity as the NIm sites have been identified [39-42].

Table 1: The genus *Enterovirus*: representative virus and the diseases that they cause

Species	Serotypes	Virus	Diseases	Ref.
HEV-A	23	CV-A2 to CV-A8, CV-A10, CV-A12, CV-A14, CV-A16, HEV71, HEV76, HEV89, HEV90, HEV91, HEV92	Hand-foot-mouth disease	[43]
HEV-B	60	CV-B1 to CV-B6, CV-A9, Echo1 to Echo7, Echo9, Echo11 to Echo21, Echo24 to Echo33, HEV69, HEV73, HEV74, HEV75, HEV77 to HEV88, HEV93, HEV97, HEV98, HEV100, HEV101, HEV106, HEV107, HEV110	Hepatitis, Encephalitis	[16]
HEV-C	23	CV-A1, CV-A11, CV-A13, CV-A17, CV-A19 to CV-A22, CV-A24, HEV95, HEV96, HEV99, HEV102, HEV104, HEV105, HEV109, HEV113, HEV116 to HEV-C118.	ARTIs	[19, 44]
		PV1 to PV3	Poliomyelitis	[17]
HEV-D	5	HEV68 (HRV87), HEV70, HEV94, HEV111	Pneumonia, Asthma exacerbation	[12, 45, 46]
HRV-A	75	HRV1, HRV2, HRV7, HRV8 to HRV13, HRV15, HRV16, HRV18 to HRV25, HRV28 to HRV34, HRV36, HRV38 to HRV41, HRV43 to HRV47, HRV49 to HRV51, HRV53 to HRV68, HRV71, HRV73 to HRV78, HRV80 to HRV82, HRV85, HRV88 to HRV90, HRV94, HRV95, HRV96, HRV98, HRV100 to HRV103	ARTIs such as common cold, pneumonia, wheezing, bronchitis, and asthma exacerbation	[47, 48]
HRV-B	25	HRV3 to HRV6, HRV14, HRV17, HRV26, HRV27, HRV35, HRV37, HRV42, HRV48, HRV52, HRV69, HRV70, HRV72, HRV79, HRV83, HRV84, HRV86, HRV91 to HRV93, HRV97, HRV99		
HRV-C	51	HRV-C1 to HRV-C51		

HRV= Human Rhinovirus/ HEV = Human Enterovirus/ PV = Poliovirus/ CV = Coxsackie virus/ Echo = Echovirus

ICAM-1 consists of 5 extracellular immunoglobulin superfamily domains denoted as D1 to D5 with a transmembrane region, and a short cytoplasmic domain (Fig 7). The 90 kDa ICAM-1 receptor plays significant roles in virus attachment, pH-internalization and uncoating processes. Binding mechanism between ICAM-1 and major-group HRVs

involves in 2 steps; (1) D1 domain of ICAM-1 penetrates primarily into the canyon floor and interacts with parts of VP1 and VP3 capsid proteins, while the CD loop of ICAM-1 lies against the 'south' rim of the canyon and (2) the binding process mediates a conformational change of the capsid, dislodging the pocket factor, and facilitate externalization of VP4 and other internal viral components including genomic RNA to host cell cytoplasm (Fig 8) [49]. The interaction between ICAM-1 and the canyon floors of HRV-A and HRV-B is species-specific due to it requires a distinct set of nonlinear amino for the receptor binding. Despite the evolutionary divergence of HRV, evolutionary analyses on the ICAM-1 binding site of HRV reveal the conservation residues among major group HRV members implying evolutionary advantage in this site [36]. The LDLR consists of 8 imperfect ligand-binding repeats of ~40 amino acids at its N-terminus, followed by an epidermal growth factor precursor domain, an O-linked sugar domain, a transmembrane segment, and a cytoplasmic domain containing coated-pit internalization signals. It is a cell surface receptors, which mediate the transport of structurally and functionally diverse protein ligands into cells by receptor-mediated endocytosis. In contrast to the major group HRV, binding of minor group HRV to LDLR occurs around the fivefold axes, not in the canyons, resulting in destabilization of vial capsid. In addition, LDLR plays roles in attachment process and internalization but not directly initiate uncoating, while the uncoating process occurs by internalization the pH-environment in host cell [50-52].

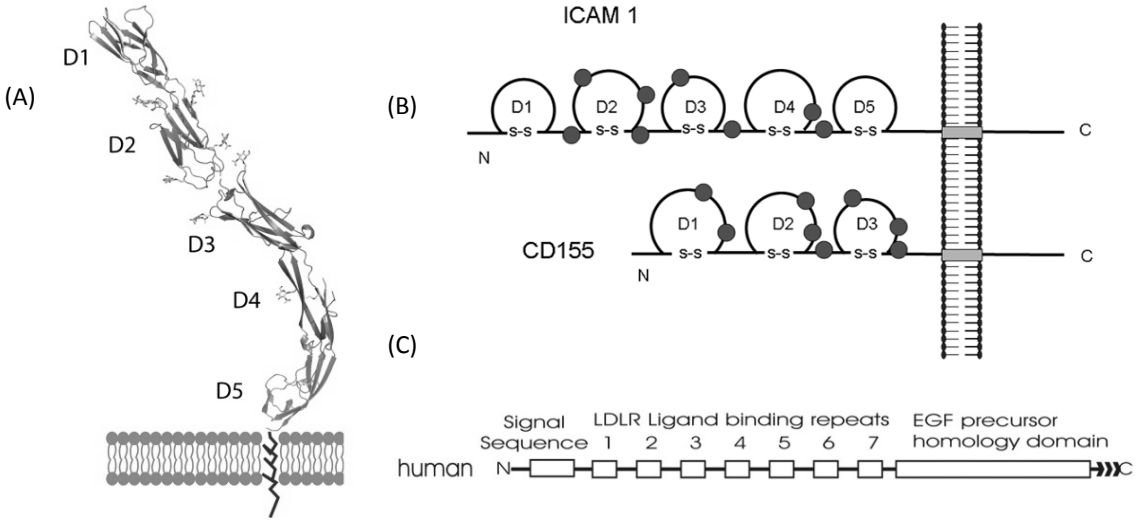

Figure 7: Diagram represents the domain of (A-B) ICAM-1 and (B) CD155 structures (modified from http://jin-lab.org/wiki/) and (C) LDLR [53].

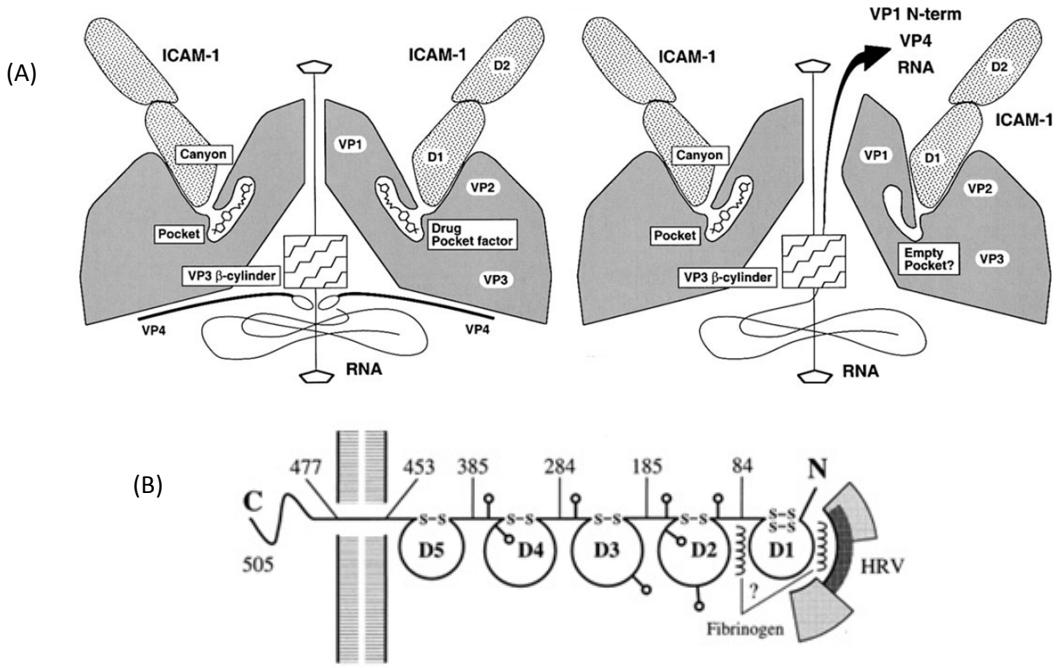

Figure 8: Schematic representation of (A) an interaction between ICAM-1 and major HRV and the mechanism of decapsidation. ICAM-1 is represented only as a two-domain fragment in this figure [54]. (B) A diagram of an ICAM-1 showing sites of glycosylation and the approximate location of binding sites of HRV [55].

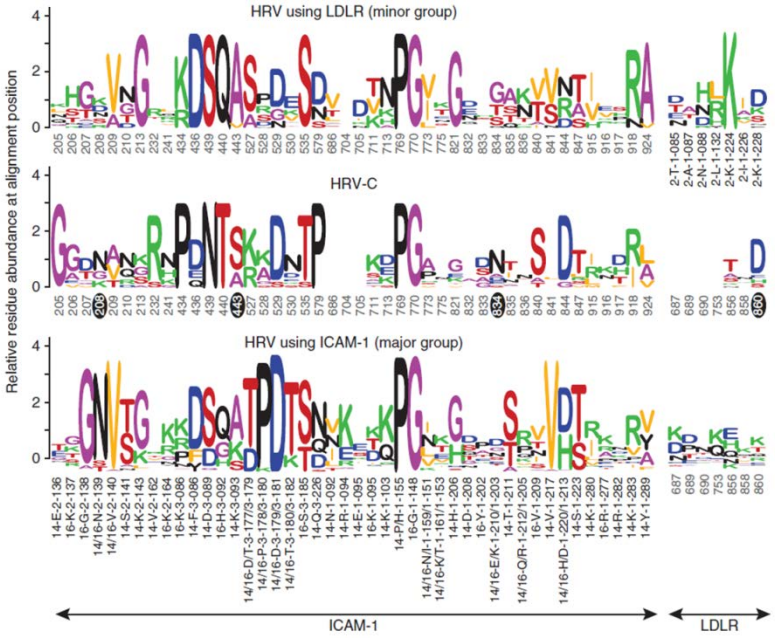

Figure 9. Schematic represents HRV-C composition compares to the ICAM- Dominant amino-acid compositions at alignment positions with ICAM-1 (HRV14 or HRV16) or LDLR (HRV2) receptor contact residues is depicted by capital letter [56].

The receptor preference and mechanism by which HRV-C utilizes for host cells entry and its pathogenicity have been undetermined due to, unlike HRV-A and HRV-B, it has not been successfully propagated in any cell culture system. Reverse genetic and

genomic sequence studies of HRV-C suggested that they have 40% footprint composition conservation in the canyon floor of VP1 protein when compares to ICAM-1 receptor binding site, while they do not have the Lys224 which is an important residue for LDLR binding suggesting little commonality between HRV-C and the ICAM-1 or LDLR interact residues [48, 54, 56, 57]. Overall the analyses evidences hypothesized that HRV-C may either utilize different receptors or use the same receptor with distinct compositional interacting footprint from HRV-A and HRV-B.

The genomes of HEV possess higher percentage of average guanine and cytosine (G+C) composition than HRV genomes which are suggested to be an essential genomic factor for the virus adaptive capability to replicate in various parts of the human body including respiratory tract, gastrointestinal tract, and central nervous system (CNS) [58]. In parallel with the molecular characteristics of HRV species, they exhibit a lower G+C content and can replicate effectively at the low temperature environment found in the upper airways (33-35^{o}C). HEV utilizes several kinds of receptors such as PV use ICAM-1 and human cluster of differentiation 155 (CD155) for PV [59] and CV and several strains of CV genotypes B1 (CV-B1), CV-B3 and CV-B5 complexes with adenovirus receptor (CAR) and/ or decay-accelerating factor (DAF) [60, 61]. Many Echo serotypes including Echo3, Echo 6, Echo 7, Echo 11, Echo 12, Echo 13, Echo 19, Echo 21, Echo 24, Echo 25, Echo 29, Echo 30, and Echo 33 also use DAF as their cellular receptor [62, 63] whereas Echo1 and Echo8 bind very late antigen (VLA-2) integrin [64]. HEV species A is one of important species utilizes broad spectrum of receptors. For example, neuropathogenic associated HEV71 and CV-A16, the causative pathogens of hand-foot and mouth disease, use 2 human membrane proteins including human scavenger receptor class B2 (SCARB2, also known as lysosomal integral membrane protein II or CD36b like-2) and P-selectin glycoprotein ligand-1 (PSGL1 or CD162) [65-69] as their functional receptors. CV-A24 uses sialic acid receptor for cell entry [70].

Most of HEV and HRV have short incubation period range from 3-10 days after infection. The primary site of HRV inoculation is the nasal mucosa, although the conjunctiva may be involved to a lesser extent. HRV is an acid intolerance virus which is sensitive to the gastrointestinal tract's acidic pH (pH<3), and this explains why HRV infection is restricted only to the respiratory tract. In contrast, acid tolerance is one of the general features of HEV. Most of HEV enter the host via the oral cavity (oral-fecal route

transmission) and respiratory tract. The replications occur mainly at lymphoid tissues of the respiratory tract (Peyer patches, mesenteric nodes, tonsils, and cervical nodes) and/ or intestinal mucosa of the gastrointestinal tract [71, 72]. Viruses then enter to the bloodstream in order to disseminate to a variety of target organs including CNS, heart, liver, pancreas, skin, and mucous membranes. Dissemination and replication of HEV in the secondary sites result in viremia which may correlate with the biphasic appearance of fever and symptoms commonly found in HEV infection. Despite HEV can efficiently replicate at the higher temperature compared to HRV (~37^{o}C), some types of HEV have general properties similar to HRV and exhibit specific respiratory tropism such as HEV68.

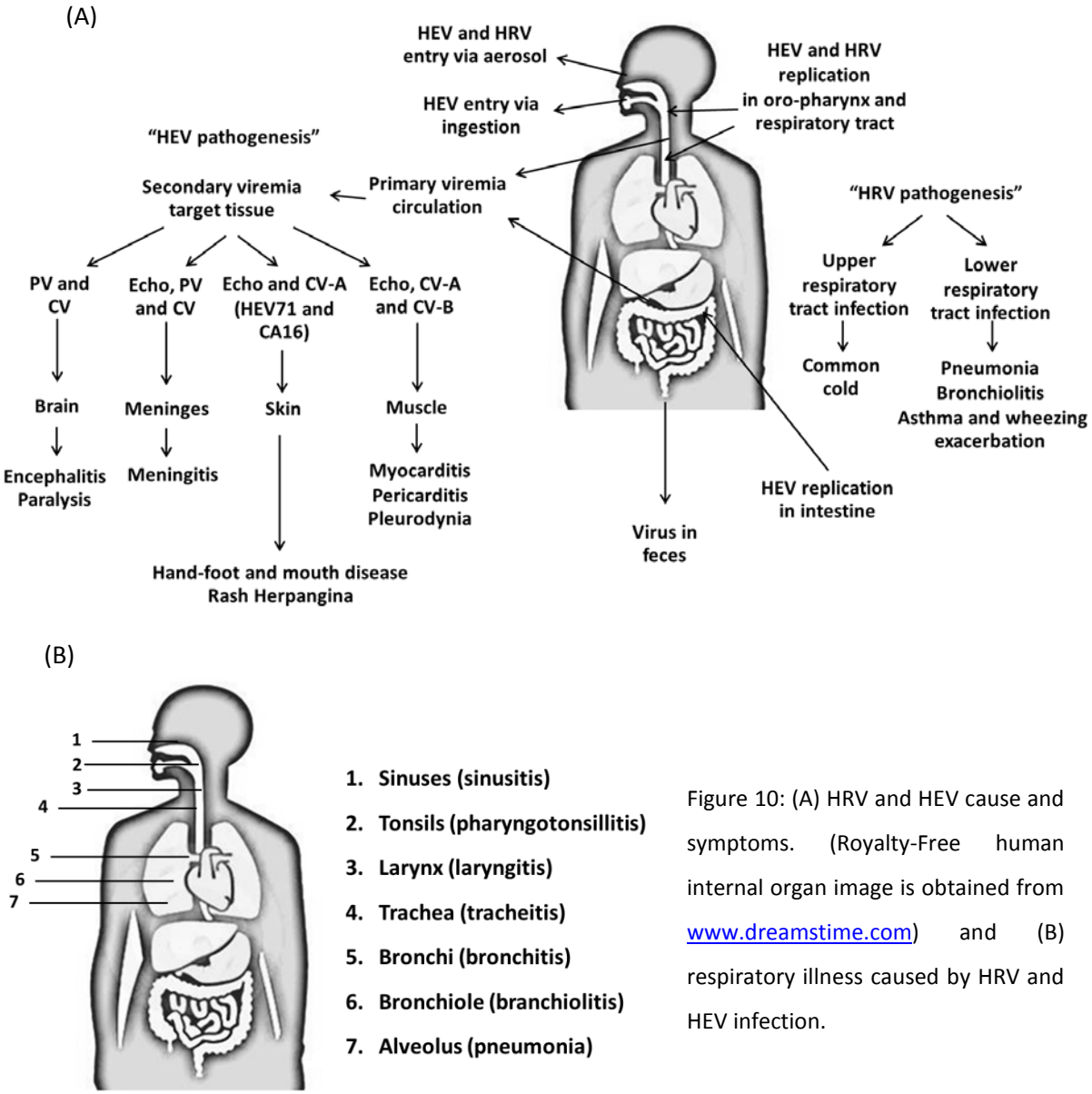

Figure 10: (A) HRV and HEV cause and symptoms. (Royalty-Free human internal organ image is obtained from www.dreamstime.com) and (B) respiratory illness caused by HRV and HEV infection.

Evolutionary mechanisms leading to genetic variability in HRV and HEV

HRV and HEV are considered as high infectivity and transmissivity pathogens. Studying of populations of the viruses shows extensive genetic and antigenic heterogeneity reflecting in the identification of >100 serotypes among them. These can result in generations of many circulating strains during their epidemic and allow for the virus adaptation dynamically to different hosts and environments.

1. Genetic diversification driven by recombination process

Recombination is a term used to describe the natural process that produces a new combination of non-segmented virus genes or genomes, derived from two parental genomes, and the new progeny that produced is referred to as a recombinant virus, while this process called as 'reassortment' in segmented genome virus. Recombination has been known to occur in the picornavirus genome as early as 1962–1963. It was firstly introduced between serotypes of PV in oral-vaccine recipients [73, 74]. Since then, similar approaches have been used to demonstrate that genomes of RNA viruses of animals, plants, and bacteria are all capable of recombination. More recombination events in human RNA virus have been observed in a wide range of circulating HEV, Ahpthovirus, and more recently Parechovirus [75-78]. In the recombination process, nucleotide sequences of the donor RNA are introduced into an acceptor molecule resulting in generations of the new RNA strand which contains genetic information combined from more than one sources. These theoretical explanations may suggest the 2 major benefits of recombination process: (1) it provides genetic variation, rapid evolution, or better phenotypic characteristics of the progeny, which results in the spread of advantageous traits such as expansion host range and increase virulence (2) it permits elimination of harmful biological properties and genome dysfunction.

1.1 Model of recombination

PV has been extensively used as a model for RNA recombination analysis. The PV model has been proven to be a useful model system for understanding of the picornavirus and other ssRNA virus molecular biology including recombination processes. There are 3 different mechanisms for RNA rearrangement have been proposed comprising the (1) 'primer alignment and extension', (2) 'breakage and ligation', and (3) 'template switch' or 'copy choice' mechanism. The primer alignment and extension mechanism depends on the hybridization of the helper RNA molecule transcribed from the parental template, and the

homologous sequence in the anti-genomic template. This resulting hybrid molecule can serve as a primer in the extension reaction to yield a recombinant genome (Fig 11) [79]. The breakage and ligation mechanism is a non-replicative RNA recombination referred to the joining of pre-synthesized RNA molecules occurred post-replicatively [80]. During the process, pre-synthesized RNA is cleaved at specific points and ligated to form a hybrid genome. Despite reasons for termination of donor strand elongation have not yet been established, several major factors have been proposed to explain non-replicative RNA recombination including enzyme mediation, and self-complementary formed of the stable RNA secondary structure.

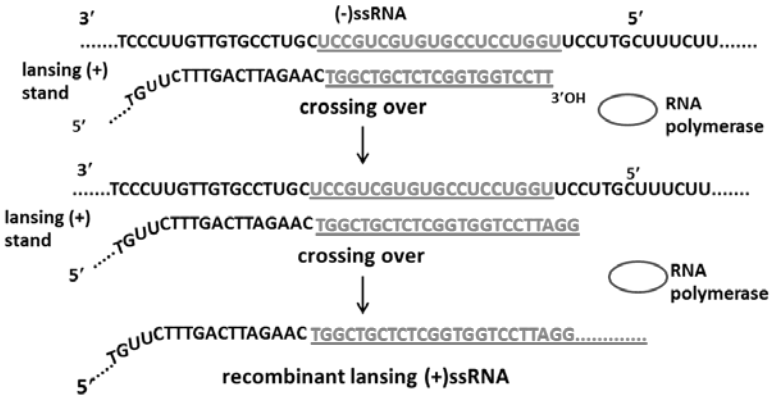

Figure 11. Primer 'Alignment-and-Extension' model.

Alternatively, the template switch is the most recently accepted mechanism of +ssRNA virus recombination (Fig 12). It is referred to the mechanism by which the RNA molecule that is synthesized on one template may be further elongated by using another template [81]. According to this model, the hybrid RNA is generated during the -ssRNA synthesis when the RdRp switches the incomplete nascent molecule from interrupted donor template to serve as a primer for resuming strand elongation on the other acceptor template, and resulting in production of a new chimeric genome. The sequence similarity between donor and acceptor molecules is thought to be the major factor mediating this process. Accordingly, the template switch recombination can be divided to 2 types; (1) homologous recombination as the process occurs most often between high sequence similarity of the two parental regions (2) non-homologous recombination as the crossover falls between genes and proteins domain with little sequence similarity or different between non-related RNA molecules. The non-homologous recombination may lead to generation of deleterious genomes' fitness.

In human DNA virus, the recombination in latent infection between the cell and viral DNA is integrated at a specific site in chromosome. In contrast to DNA virus, variations by sharing of RNA virus genes occur randomly sequence similarity of the participating molecule. The picornavirus recombinations occurred through single-crossing over of the different RNA templates with breakpoint positions generally encompass in the 5′UTR, 2A, 2C, and 3D [82]. The distribution of these certain types of recombination breakpoints identified in both of clinical strains and *in vitro* models may not be fortuitous, and may be promoted by stable stem-loop structures which are identical between the recombining partners [83]. These suggested that the recombination frequency explained by the template switch mechanism is relied on the sequence similarity of the participating molecule.

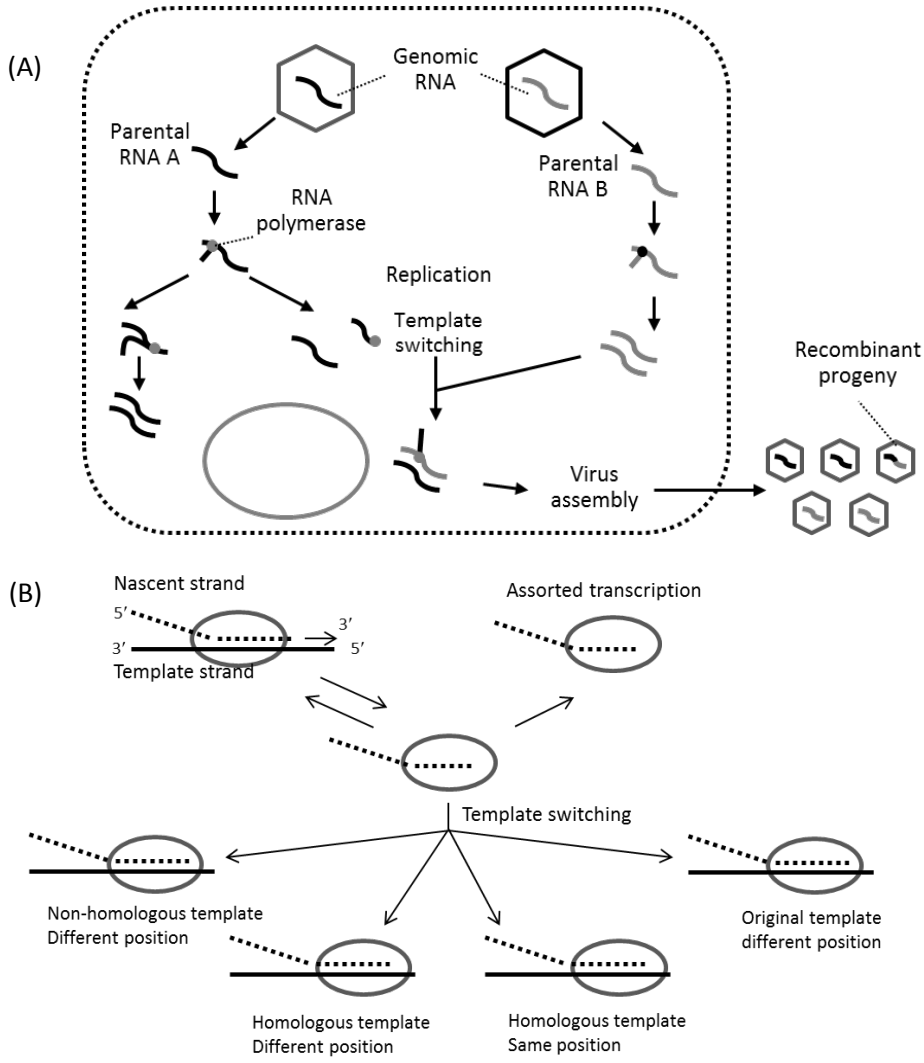

Figure 12. Template switch model; (A) Co-infection between two or more distinct virus strains can be lead to the generation of recombinant virus. (B) Homologous and non-homologous recombination [84].

The evolutionary forces driving the diversity in HRV appear to contradict from other picornaviruses where the recombination has recently been proposed to play significant roles in genetic diversification [85, 86]. Results from earlier analysis at the genome level by using the sequence dataset covered all of HRV references showed the consistent of phylogenetic tree topologies and levels of sequence diversity across the genomes [28]. Despite the receptor binding preferences between ICAM-1 and LDLR apparently presented no barriers to genetic exchange, recombination events occur infrequently among HRV-A and HRV-B members. These raise the possibility that the recombination process may not be the major driving force of diversification of HRV [28, 87]. Recent bioinformatics studies have shown that all of recombination events detected in HRV-A and HRV-B were small in sizes and predicted as the consequence of the double-cross over mechanism [87]. Recombination breakpoints occurs primarily at the area adjacent to the beginning of the ORF (5'UTR), and a few distinct loci scattered throughout the coding region such as boundary between the capsid VP1 and non-structural genes or extending into the 2B gene [85]. Despite recombination occurs frequently in some of picornavirus genera, not all of the chimeric viruses can survive. In some cases, generation of recombinant-selection reduces the progeny chimera to outcompete the parental genomes' fitness and influences on the protein function.

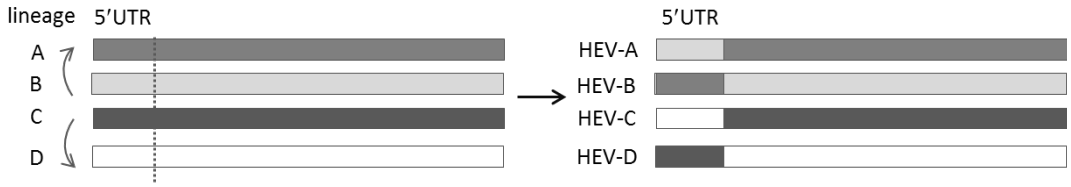

Figure 13. Schematic representation of proposed recombination events between the 5'UTR and the rest of the genome during HRV evolution.

HEV are known for high genome plasticity and genetic diversity, due to both high mutation rate and recombination within and between serotypes. Numbers of studies have been reported that recombination is extremely frequent in HEV. In the majority of recombination cases of HEV have been shown to take place strictly between members of the same species, and usually spare the 5'UTR and P1 region (Fig 13) [86, 88, 89]. Recombination process generates the new HEV progenies that contribute to the development of many diseases. For example, HEV109 is a new interspecies recombinant

HEV derived from genetic exchanges between 5'UTR of HEV-A and the remaining genome parts from HEV-C [44]. Infections by HEV109 have been reported to associate with many diseases including acute respiratory tract illness [90], acute flaccid paralysis [91],

1.2. Rate of recombination

Recombination rate is not a selectively determined trait in itself but rather the natural outcome of particular genome structure or virus ecology. The rates of recombination in RNA viruses can be measured in 2 different methods; (1) the rate of template switching that occurs during replication (2) recombination rate amongst the population level. The rates of recombination are highly variable in RNA viruses. For example, recombination appears to occur frequently in some viruses such as retrovirus-human immunodeficiency virus (HIV) which has an estimated recombination rate between 1.38×10^{-4} and 1.4×10^{-5}/ site/ generation and 2-3 times switches templates when the entire genomes have been transcribed [92, 93] and approximately 7×10^{-3} to 8×10^{-3}/ lineage/ year [94-96] of intersubtype recombination; on some +ssRNA virus such as picornavirus (HEV and PV), coronavirus and some of segmented -ssRNA virus such as Flu-A has an average of one re-assortment every 2-3 years [97].

2. Genetic diversification driven by mutation

HRV species B (serotype 14)																									
amino acid position	104	105	106	107	124	126	128	130	150	151	152	174	175	176	186	188	191	197	199	215	219	221	224	245	267
majority sequence	I	N	L	S	F	S	Y	I	A	M	Y	P	S	V	F	V	V	Y	C	I	N	M	M	H	G
26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	F	-	-	-	-	-	L	-	-	-	-	-	-	-	-
42	-	-	-	-	-	-	-	-	-	-	F	-	-	-	-	-	L	-	-	-	-	-	-	-	-
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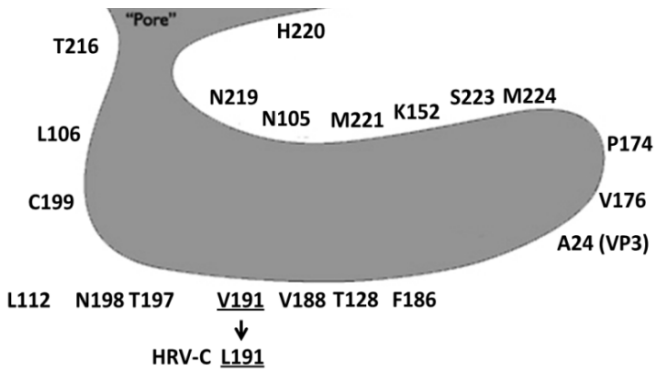


Figure 14. Alignment in the binding pocket of HRV-C compares to the representative of antiviral resistance HRV-B. The amino acids chosen for this analysis are the residues within 4 Å of a collection of related antiviral compounds as determined by X-ray crystallography in HRV-14 [98], references of serotype of HRV-B. HRV-B viruses are listed by order of decreasing susceptibility to pleconaril.

Mutation in virus gene and genome has been proposed to be a significant factor underlying on the HRV and HEV diversifications, which could continually lead to emergence of the new strains. As with all RNA viruses, point mutation is introduced into the viral genome by errors in replication process which are driven by several factors including the replication fidelity, high error-prone RdRp combined with the absence of proofreading and post-replicative repair mechanisms. These factors increase the number of mutations incorporated into the viral genomes overtime with the frequency of 1 per 10^3 to 10^4 nucleotides per replication cycle [99], and prepare the ground for rapid genetic diversifications over the short period of infection time. This frequency may far exceed the corresponding rate of DNA viruses which represent ~1 per 10^8 to 10^{10} substitutions per nucleotide copied. Furthermore, genetic variations in RNA viruses can also be increased by selection mechanisms in order to control their replication capacity from several pressures such as host immune defense, vaccination, antiviral treatment, and competition among co-circulating viruses [86, 100]. These variations result in either direct or indirect alter antigenic properties of the viruses and also impact on fitness and virulence of the viruses in order to evade host's immune responses. The external surface of the capsid proteins, particularly in regions directly involved in antibody binding and host cellular receptor binding, of some viruses such as picornaviruses have been suggested to be high genetically diverse regions. Furthermore, accumulation of mutation in the subsurface binding hydrophobic pocket located beneath the 'canyon' of capsid and can lead to antiviral resistance adaptation in picornaviruses. Mutation in the binding pocket affects many biochemical properties of amino acid which may in turn affect the binding affinity of pleconaril antiviral drug such as changing the hydroxyl function in that region of the pocket and enhancing conformation flexibility [101]. For example, virus isolation from recovered patients suggested that pleconaril antiviral resistance in 7 serotypes of HRV-B which all share Phe152 and Leu191 residue pattern in the pocket, have been associated with the accumulation of single point mutation in VP1 gene. Moreover, the observations in HRV-C's sequence characteristic suggested that some HRV-C strains have amino acid substitutions in the same positions (L191) and may confer natural phenotypic resistance or weak susceptible to pleconaril similar to that HRV-B [22, 48, 101]. After generation of mutant genomes, the processes that define the fate of new genotypes are genetic drift and diversifying selection. In general, neutral mutations can become fixed in the population by

genetic drift. The term of genetic drift can include fixation, or elimination of the mutations. However, if a mutation changes the fitness of an organism, directionally selection will act on the mutation in addition to drift. Diversifying selection can be divided into 3 types based on the consequent caused by mutation. Positive selection acts to increase the frequency of advantageous mutations. Negative or purifying selection acts to decrease the frequency of deleterious mutations.

Epidemiological characteristics of HRV and HEV68

The new human rhinovirus species, HRV-C

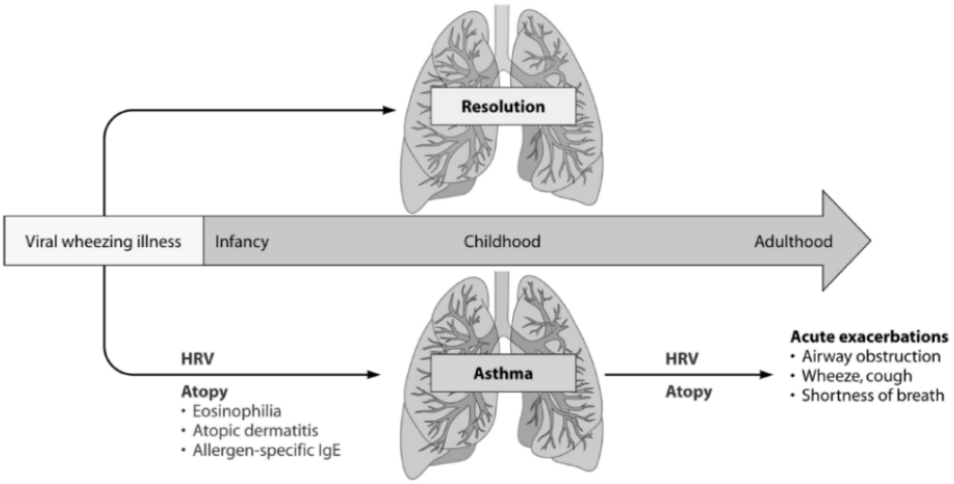


Figure 15: The relationship between HRV infections and asthma.

Numbers of respiratory surveillance network have reported that HRV is predominantly found in hospital-based pediatric patients with pneumonia, bronchiolitis, wheezing, and chronic respiratory disease exacerbation. In infancy, illnesses such as bronchiolitis share common clinical characteristics with acute asthma including wheezing, rapid breathing, prolong small airway inflammation, and respiratory compromise [102]. Furthermore, long-term studies have demonstrated that infants who have acute wheezing illnesses with virus infections undergo the development of recurrent wheezing, and these infants may have greater risks for subsequent asthma. Interestingly, reports showed that approximately two-third of them were caused by HRV [103]. They were not only suffering from greater disease severity but also prolonged lower respiratory symptoms more often than in subjects unaffected by asthma. Moreover, similar findings have also been observed for adults hospitalized with acute asthma. Series of epidemiological studies published

between 2006 and 2007 described a novel species of HRV, referred to HRV-C or A2 group, which had genetically distinct from previous species.

HRV-C had been detected with distinct genetic and biological characteristics including the genome size, location of *cis*-replicating element, receptor specificity, genetic evolution and variability [28, 48] and might not be susceptible to appropriate antibiotic treatment [104]. Furthermore, some of HRV-C variants showed phylogenetic compatibility and high sequence similarity in 5'UTR when compared to HRV-A while the remaining genome parts showed phylogenetically distinct features from other species [105, 106]. This implied that those HRV-C variants genetically interspecies recombined with HRV-A variants, with recombination breakpoints located in the stem-loop 5 and polypyrimidine tract of, resulting in classification of 2 separate clusters of HRV-C, Ca and Cc (Fig 15) [107].

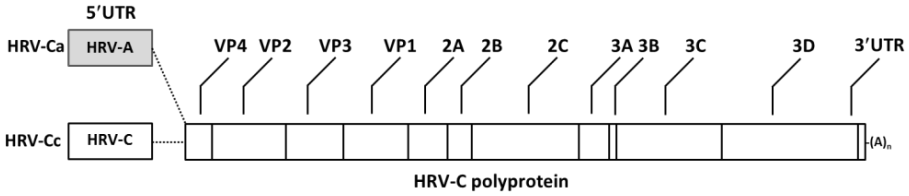


Figure 16: Schematic representation of the HRV-C genomes and the 5'UTR origin.

Interchangeability of in 5'UTR between HRV and HEV were experimentally proven by generating the chimeric of 2 different clinical HRV-C (HRV-Ca and HRV-Cc), and HRV-B strain or HEV-A strain, and the remaining part of the genome from an HRV-A [108]. The result showed that the chimeric virus was easily propagated in cell culture as similar as the wild type viruses, and did not affect its functional RNA structural elements. HRV-C has been recognized as globally distributed viruses and frequently associated with more severe respiratory diseases compared with other species [109, 110]. However, the inability to grow HRV-C in tissue culture has limited the understanding of their pathogenicity and the mechanisms of host immune response to HRV-C infection. Furthermore, diagnoses of respiratory infection were usually based on clinical and epidemiological criteria which included only severe cases. Consequently, the data investigated on patients admitted to hospital so far has remained limited.

The remerged human enterovirus, HEV68

HEV68 is a sporadically detected viral pathogen associated with respiratory tract illnesses with various clinical consequences ranging from mild to severe respiratory diseases including wheezing, pneumonia and bronchiolitis [11-13, 111]. HEV68 belongs to the genus *Enterovirus* species D which currently comprises 2 important human pathogens, HEV70 and HEV94. It possesses a +ssRNA of ~7.5 kb in length. In contrast to other HEV, HEV68 shares common biological properties with HRV, which is acid sensitive virus and grow efficiently at an optimum temperature of ~33^{o}C [112, 113]. However, it is unique in its receptor usage. Infection by HEV68 requires the presence of sialic acid on cellular receptor or decay-accelerating factor, which is known to be the receptor of HEV70 [112]. Decay-accelerating factor (DEF) is a 70 kDa glycophosphatidyl inositol-anchored glycoprotein encoded by CD55 gene which is located in the complement regulatory locus on the long arm of the chromosome 1. DEF functions in regulating of the complement system in cell surface, and inhibits analogous complement cascade activation (formation of C3/C5 convertase) in order to avoid the cell destruction and promote the C3 and C5 complex metabolism. DEF is found expressed on the most cells surface such as erythrocytes and on cells with close contact to the serum complement including palettes, monocytes, leucocytes, endothelial cells and epithelium of the urogenital tract and gastrointestinal tract. DEF composes of 4 contiguous short consensus repeats (SCR; 60-70 amino acid in \repeating unit) at N-terminus and the active site for complement C3/C4 is located in within this structure. The SCR domains are connected to a serine-threonine-rich region and linked to cell membrane via glycosyl-phosphatidy-linositol anchor.

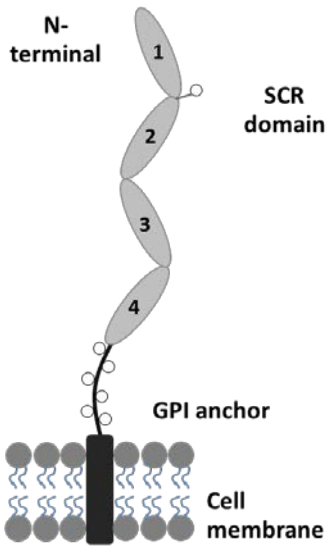


Figure 17. Schematic representation of decay-accelerating factor receptor

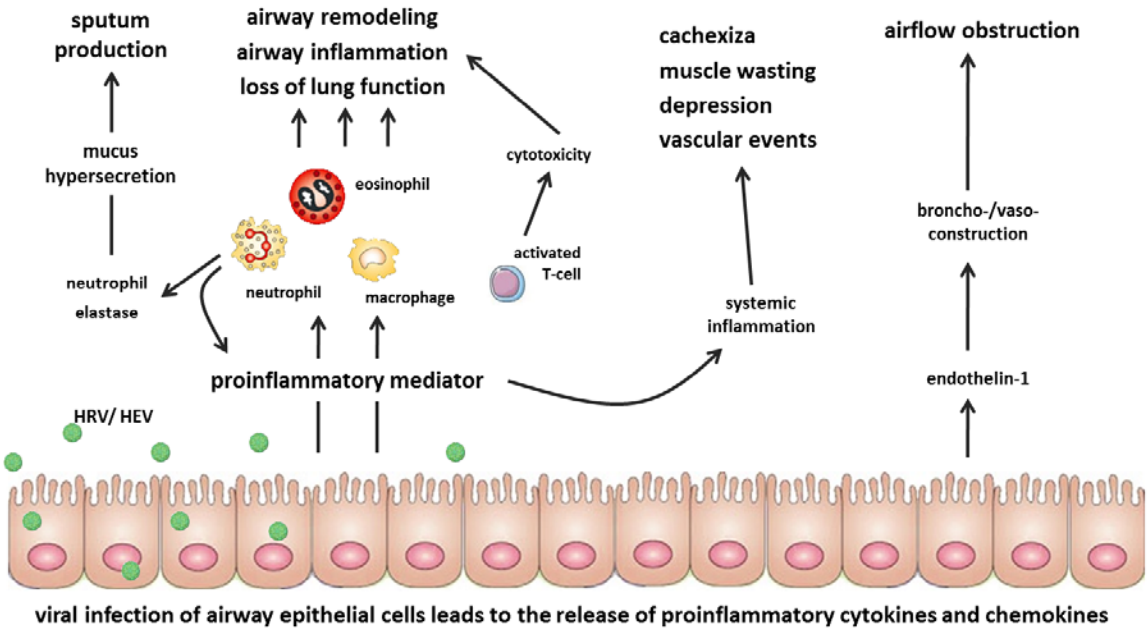
Since HEV68 was first isolated in California in 1962 from children with pneumonia and bronchiolitis by using virus isolation and serotyping approaches [114], limited numbers of epidemiological profile of HEV68 and its pathogenicity have been reported. From 1970 to 2005, only 26 HEV68 clinical isolates were detected in the United State [115].

Longitudinal study in the Netherland during 1994 through 2010 showed only 0.5% annual prevalence of HEV68 (71 of 13310 cases) and all of which were obtained from respiratory specimens [116]. HEV68 has been regarded as an uncommon pathogen. The involvements of HEV68 in CNS disease have also been reported by various US research teams. Khetsuriani and colleagues discovered the virus from an acute flaccid paralysis case [115], while the other group found HEV68 in cerebrospinal fluid samples taken from fatal meningomyeloencephalitis children [46]. However, the full spectrum of illness caused by HEV68 infection still remains unclear. Clusters of the rare HEV68 have been reported more frequently after the year of 2008 suggesting the re-occurrence of HEV68 in several countries worldwide. However, since 2008, only 4 studies have been reported from the Asian continent. Among these, 2 studies were reported from Japan during the period of 2006-2009 and 2009-2010, and the other two were reported from the Philippines and China during the period of 2009-2010 and 2006-2010, respectively [11-13, 117]. Importantly, 3 fatal cases have been found in these studies: 2 cases from the Philippines and 1 case from Japan.

Host cellular response to HRV and HEV infection

The bronchial epithelium playing a provital role in innate immunity as it provides barrier between the respiratory pathogens and internal parenchyma. Infections by HRV and HEV mediate a wide range of secreted mediators including pro-inflammatory cytokines, chemokines, interferons (e.g. IL-1, IL-6, IL8, CXCL8/IL-8, IP-10, and CCL5), and growth factors [118, 119]. These mediators effect to the inflammation of eosinophil, neutrophil, and lymphocyte as well as mucus hyper-secretion, airway remodeling, and hyperinflation of lung tissue (Fig 18 and appendix A). Adaptive immunity to the virus infection involves an early immunoglobulin M (IgM) antibody response at 7-10 days post infection, followed by a rise in IgG and intestinal IgA neutralizing antibodies (Ab) response at 14 days (Fig 18). In case of reinfection occurs with the same serotype, specific Ab raised after the previous infection will play crucial role in order to ameliorate the symptoms and, therefore, the illnesses may be subclinical. However, the possession of specific Ab is not sufficient to provide heteroserotype cross-protection and disease reduction during the episode of different type of the virus re-infection. As epidemiology behaviors of some HRV and HEV strains, long-term individuals' continuity or repeated infections with the same

serotype of the virus has been occurred infrequently [120]. Hypothesis describes about the high incidence of repeat infection phenomenon of HRV and HEV in individuals might be associated with the relative inability of specific Ab in response to the elimination of viral replication resulting on the development of severe diseases. Furthermore, one of the reasons why Infections by HEV and HRV in infants are the higher risk for clinical complication than other age group can also be described by lack of maternal passive specific Ab and inability to induce a significant adaptive immune response.



viral infection of airway epithelial cells leads to the release of proinflammatory cytokines and chemokines

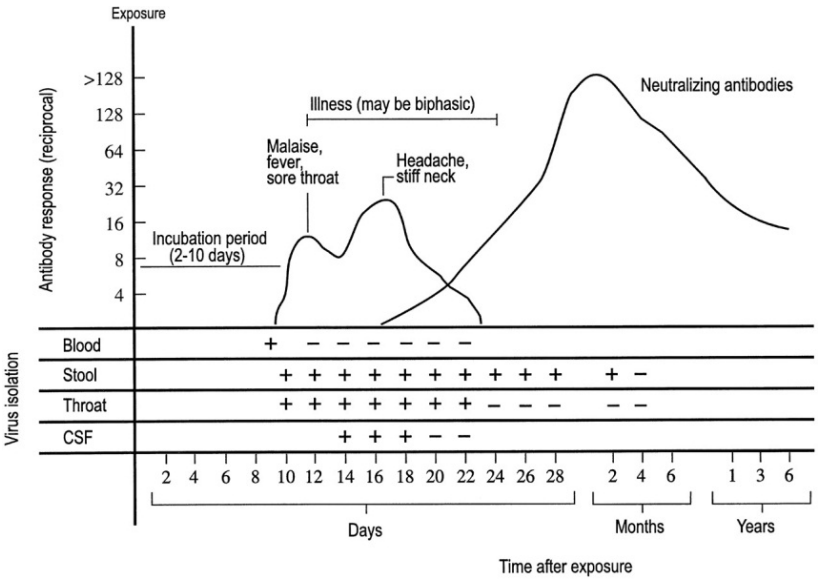


Figure 18. (A) Scheme of the innate immune response phases against HRV and HEV infection. (B) Clinical manifestation, viral isolation, and adaptive antibody response in non-polio enteroviral infection [71].

However, in most cases, the titers of neutralizing Ab are not able to be detected until 14 days after infection. Moreover, resistances to HRV infection and disease

progression in patients have been found to associate with high pre-existing neutralizing Ab titers [121]. Therefore, recovery from the respiratory tract illnesses within the short period of time is probably mediated by other agents such as host produced microRNA.

MiRNA function in response to eliminate the virus infection

As a group of newly identified cellular regulators, microRNA (miRNA) has been known as a key regulator to modulate the expressions of diverse array of cellular genes and several biological processes including cellular proliferation, apoptosis, homeostasis, and tumor formation by posttranslational regulations of target messenger RNA. It is a recently discovered class of short non-protein coding RNA of 21-23 nt. More details about miRNA and its regulation mechanism are summarized in appendix B. MiRNAs contribute to the regulation of the picornavirus life-cycle at multiple levels, including causing the catalytic degradation of the viral genome, and interfere with genome encapsidation. The viruses may promote their life cycle by modulating the intracellular environments as it has been shown that upregulation of miR-141 in response to HEV71, CV-B3 and PV3 infections could facilitate viral propagations by cleavage of host translational initiation factor eIF4E. The eIF4E is a canonical initiation factor required for cap-dependent translation [122]. In addition, cleavage of eIF4E contributes to virus-mediated host protein synthesis shutoff and expedites the translational switch from cap-dependent to IRES-dependent translation. *In vitro* study of miRNA expression profiling upon RV infection was investigated by Ouda and colleagues. They demonstrated that cellular miRNA expression can be modulated during HRV infection with irrespective of the virulence of the virus. Of all unregulated miRNAs, miR-23b targets the cellular mRNA encoding a cell-surface protein VLDL. VLDL is known to be a common receptor utilized by HRV minor group, suggesting that upregulation of endogenous miR-23b was significant for host defense to the minor group HRV infection. However, they only showed results in the host systemic response to viral infection. Therefore, the interaction of host miRNAs that directly targets to viral genome is remains poorly understood and warrants further studies.

CHAPTER III

EXPERIMENTAL RESULTS

Part 1: Molecular epidemiological studies of HRV and HEV68 among pediatric patients with acute respiratory tract illnesses in Bangkok, Thailand.

To date, data on epidemic and seasonality of respiratory viruses associated ARTIs have been reported in several countries worldwide with distinct pattern between temperate and tropical region [116, 123-126]. In temperate climate regions, clear seasonal pattern variations of ARTI occurrences are obviously observed. The epidemic patterns have been frequently observed during the winter months and early spring. Tropical climate condition is characterized by high average temperature along with profound humidity throughout year and torrential rain during the rainy season. As the consequence of the less change in seasonal temperatures, the less distinct of ARTI seasonality with some local variation has been found in these areas including Thailand. In Thailand, >40% of the total number of pediatric ARTI cases are outpatients [127]. Although numbers of studies have showed the characteristics of various types of respiratory viruses in Thailand [128-130] and that could increase the knowledge on virus epidemics, data on specific etiological agents in large cohort population and long-term follow up analysis are still lacking. Furthermore, studies on epidemiology and seasonality of respiratory virus infections that lead to ARTIs should be investigated in order to establish the preventive strategies, and to develop broadly effective vaccines or antiviral drugs. Moreover, such information may give a useful predictive model for respiratory virus activities in the era of climate conditions changing worldwide. Thereby to address these concerns, this study purposed to gauge the genetic characteristics and clinical impacts of HRV infections among a large number of ARTI-children aged 15 years (yrs.) and below in Thailand during 2006-2012. Parallel screening for HEV68 was also performed for better understands of the epidemic status of this virus and its related pathogenicity. To further explore the possible impact of meteorological factors on HRV and the sporadically detected HEV68 activity, this study aimed to establish a seasonal model that can simulate the oscillation of the number of ARTI and HRV cases with weather conditions in tropical climate using the data of Bangkok, Thailand, as reference.

Research questions

1. What are the incidences of HRV and HEV68 infections in ARTI among pediatric patients?
2. What is the association between the genetic relationship and phylogenetic evolution among HRV and HEV68 strains circulating in Thailand or other countries?
3. Do meteorological factors affect HRV and HEV68 seasonal activities in Thailand?

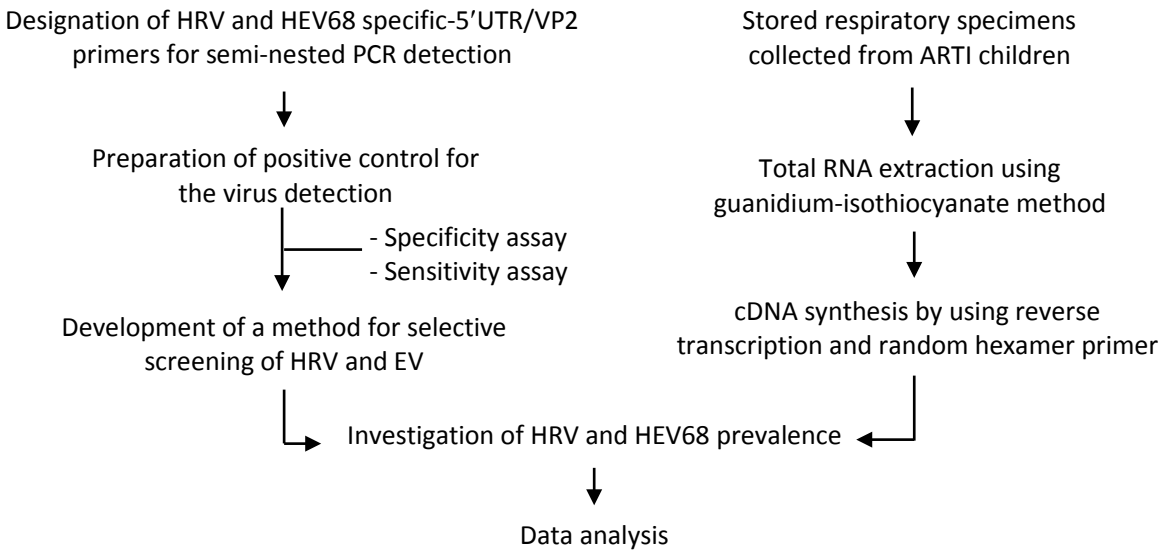
Objectives

1. To evaluate the burden of HRV and HEV68 associated to respiratory illnesses in pediatric patients and establish epidemiological profiles of these viruses in Thailand.
2. To investigate the genetic diversities and evolutional trends of HRV and HEV68.
3. To determine whether meteorological factors are associated with HRV and HEV68 epidemics among children with ARTI.

Expected benefits and applications

1. These informative data will provide major implications on the pathogenic roles of both HRV and HEV68 in respiratory diseases.
2. The nucleotide sequences of both HRV and HEV68 obtained from this study part will be submitted and served as a database for epidemiology and phylogenetic analysis.
3. Establishment of an effective model for predicting respiratory illness by using weather forecast data.

Conceptual framework



Materials and Methods

Population study

A longitudinal population-based study will be performed by using anonymous stored respiratory specimens which are available from routine respiratory viruses diagnostic testing and keep at the Center of Excellent in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok Thailand. Respiratory specimens including oropharyngeal and nasal swabs and nasopharyngeal aspirations were obtained from pediatric patients aged less than 15 yrs who had ARTI and visited to several hospitals located in Bangkok during February 14, 2006 to 2012. To assess the distribution of respiratory virus infection with regard to the specific children's age, the subjects were categorized into 4 groups as follows: infant (≤ 2 yrs), pre-school children (>2 to 5 yrs), primary-school children (>5 to 12 yrs), and secondary school children (>12 to 15 yrs).

Sample size estimation

The prevalence of HRV and HEV68 infection in children has been reported in several countries and years with variation considerably. As for examples, the HRV prevalence values are as follows: Osaka, Japan, 31%; the Philippines, 30%; the United State, 16%; Edinburg, Scotland, 24% [110, 131-133]. For EV68, 3.4% annual prevalence was reported from the Japan cluster and 2.6% from the Philippine [13, 111]. With regard to the precise of identifying epidemic data, average values of the prevalence of HRV and HEV68 in children with ARTIs will be used in the experimental designs, 25.2% and 3% for HRV and EV68, respectively. The sample size required will be calculated according to the following formula:

Description:

$$n = \frac{Z_\alpha^2 P(1-P)}{e^2}$$

- n = required sample size
- Z_α = confidence level at 95% (standard Z value of 1.96(two-tail))
- P = Incidence proportion HRV or HEV68 infection in pediatric patient with ARTIs estimated from the previous study
- e = acceptable margin of error at 3% (standard value of 0.03)

Therefore, 805 and 84 respiratory specimens will be required for the HRV and HEV68 surveillance, respectively, in order to achieve the statistically supported results with 3% acceptable error. However, by the fact that, HEV68 is a rare pathogen which can

be detected only in 3% annually, large number of sample size should be required for measurement of the virus's epidemic with higher precision.

Ethic consideration

The research protocol was approved by the Institutional Review Board (IRB number 329/54), Faculty of Medicine, Chulalongkorn University, Thailand and the need for consent were waived because the samples were de-identified. This study was conducted on specimens collected upon conclusion of routine examinations and stored as anonymous. All stored samples in this study had been gotten the using permission from the Director of Chulalongkorn King Memorial hospital. Patient identifiers including personal information (name, address) and hospitalization number were removed from these samples to protect patient confidentiality and also did not appear in any part of document in this study.

Sample collection and viral nucleic acid preparation

Posterior oropharyngeal and nasal swab specimens were collected from non-hospitalized patients who had been diagnosed with ILI. Nasopharyngeal aspirations were collected mainly from immunocompromised patients with ALRTI complications and required hospitalizations. A case of ILI was defined according to PAHO/CDC guidelines for influenza surveillance as a progression of fever (>38°C) and either a symptom of cough, sore throat or pharyngitis. Inclusion and exclusion criteria for ALRTI patient enrolment have been described previously [10]. All samples were collected in viral transport media with the addition of antibiotics ($2x10^6$ U/L of Penicillin G and 200 mg/l of Streptomycin) and transported within 48 hours to the center. All respiratory specimens were divided into aliquots and stored at -70^oC until further tested. Children admitted multiple times with more than one month between visits were considered as separate illness episodes.

Viral nucleic acid was extracted from clinical samples using a 96-well viral nucleic acid extraction kit (RBC Bioscience, Taiwan). cDNA synthesis was carried out with the MMLV reverse-transcription system (Promega, Madison,WI) and random hexamer.

Laboratory diagnosis for virus-associated ARTI

Laboratory diagnosis for virus-associated ARTI was performed by using polymerase chain reaction (PCR) amplification. Each specimen was tested for common respiratory viruses including RSV-A and RSV-B, Flu-A (pH1N1/2009, seasonal H3 and H1) and Flu-B

[134]. A semi-nested PCR assay was also used to investigate RSV type A and B infection in the respiratory specimens. Specific primers for RSV amplifications were shown in Table S6, Appendix D. For HRV-HEV screening, semi-nested PCR using a primer set covering a highly conserved 5′UTR up to and including the 5′ terminus of the capsid VP2 gene (5′UTR/VP2). The final PCR product of the specimens in which HRV implicated was ~540 nt in length. According to the close relationship between HRV and HEV genome sequences, samples that contained HEV could be identified ~650 nt. The PCR products were purified using the PCRExtract & GelExtract Mini Kits (5PRIME, Germany) and sequenced bi-directionally by automated sequencer at First BASE Laboratories Sdn Bhd (Selangor Darul Ehsan, Malaysia) to elucidate many aspects of the epidemiology and evolution of the virus. HEV68 implicated specimens were confirmed by performing PCR amplifications of the VP1 gene as previously described [45].

Genetic characterization of HRV and HEV variants

Sequence data for each clinical strain was formatted and assembled by Seqman program of DNASTAR. To investigate the genetic variability and relationship of HRV variants identified in this study compared to the previous defined types deposited in GenBank database, neighbor-joining phylogenetic analysis was performed. The 470-nucleotide sequences of the 5'UTR/VP2 were aligned using the ClustalW-based alignment as implemented in the BioEdit program. Phylogenetic trees were constructed using the neighbor-joining (NJ) method and the reliability of the NJ tree was estimated using 1000 bootstrap pseudo-replications resampling method implemented in the MEGA (v5). Phylogenetic distance among sequences was measured using Kimura's two-parameter model. All of newly identified nucleotide sequences in 5′UTR/VP2 or VP4/VP2 obtained in this study have been published in the GenBank database under accession numbers FJ435235-FJ435337, JN415562-JN415630 and KC412912-KC413018 for HRV and JQ411761-JQ411800 for HEV68. The VP1 sequences of HEV68 have been deposited in GenBank under accession numbers JQ411801- JQ411811.

Meteorological data

The climate of Thailand is tropical savanna with 3 different seasons including winter (mid-November to mid-February), summer (mid-February to mid-May), and rainy (mid-May to mid-October). Bangkok meteorological data during the study period were

provided by the Thailand Meteorology Department (http://www.tmd.go.th/en/). Meteorological data including temperature (degree Celsius (^{o}C)), rainfall (millimeter), and relative humidity (%) were routinely measured at 3-hour intervals in the Bangkok metropolis (standard code 455201) at latitude 13.43.35°N and longitude 100.33.36°E. Average values of these meteorological factors per 2 weeks were calculated in order to perform the analysis.

Statistical analysis

The descriptive statistics using the values of mean±standard deviation and categorical data were reported as percentages for frequency analysis. Statistical data comparisons between various factors (e.g. seasonality of HRV) were analyzed by means of Pearson χ^2, unpaired T-test, or Fisher's exact test as appropriate, using SPSS software (v17.0) (Chicago, USA). Bivariate analysis using 2-tailed spearman's correlation coefficient (r_s) and multiple regression analysis were performed to determine whether the meteorological factors have an impact on or are associated with HRV and HEV68 emergences in Thailand. Meteorological factors were incorporated as independent variables. All statistics tests were 2-tailed and analysis results were considered statistically significant at a $p < 0.05$.

Results

Population study

Over a 7-year study, a total of 2083 samples including nasal and throat swabs and nasopharyngeal aspirations were collected from 2055 hospitalized and non-hospitalized children (≤ 15yrs) who had attended medical centers and hospitals located in Bangkok and had been diagnosed as ARTIs. The respiratory samples were transported to the center for routine respiratory virus screening. In addition, 748 (35.9%) nasopharyngeal aspiration samples were collected from pediatric patients with LRTIs necessitating hospitalization. The remaining samples comprised 1335 (64.1%) nasal and throat swab samples which had been collected from both hospitalized and non-hospitalized patients having presented with influenza-like illness symptoms. The characteristics of all populations, study periods and type of respiratory specimens are summarized in the Table 2. Of these eligible specimens, 24 originated from 12 children and collected in the course of the same hospitalization for treatment follow-up. Twenty-five children had multiple

hospitalizations; 3 had 3 admissions and the remaining patients had 2 admissions. Of these, 43 comprising 28 of LRTI and 15 of URTI subjects could not provide specific information with regard to age resulting in the total number of 43.3% infants, 22.8% pre-school children, 29.4% elementary school children, and 9% high school children. The age distribution of enrolled subjects was between 1 d and 15 yrs.

Table 2: Main characteristics of the study populations and respiratory specimens

Study information		**Type of specimen**	**Infant (N = 884)**	**Preschool (N = 465)**	**Elementary school (N = 508)**	**High school (N = 183)**
Study period	**Patient characteristic**					
Feb 2006 to Jul 2008	LRTI complication	NP	N = 596/ 0.92±0.58	N = 76/ 3.57±0.78	N = 40/ 8.6±1.96	N = 8/ 13.4±0.48
Jul 2010 to Jul 2012						
Jun 2009 to Jul 2012	Hospitalized and non-hospitalized patients with ILI	NS	N = 288/ 1.43±0.56	N = 389/ 3.85±0.79	N = 468/ 8.6±1.96	N = 175/ 14.04±0.84
Total			1.09±0.62	3.80±0.80	8.6±1.96	14.01±0.85

LRTI = lower respiratory tract illness/ ILI = influenza-like illness/ NP = Nasopharyngeal aspiration/ NS = Nasal swab/ Age group; infant ≤2yrs, pre-school children >2-5yrs, elementary school children >5-12yrs and high school children >12-15yrs

Clinical severity of ARTI cases was defined as follows: pediatric patient with ARTI complications not requiring hospitalization and O_2 supplementation and normal to slightly increased respiratory rate was defined as 'mild' case, patient with ARTI symptoms, requiring hospitalization, and either did or did not require supplemental O_2 was defined as 'moderate' case, patient with severe respiratory failure with O_2 saturation <90%, requiring medical ventilation or O_2 supplementation, and admission to the Intensive Care Unit (ICU) was defined as 'severe' case. Consequently, 35.1% (n=732), and 0.77% (n=16) of the entire study population were considered as moderate and severe cases, respectively, while the remaining cases were mild ARTIs (64.1%; n = 1335).

HRV and its relative frequency with other respiratory viruses

Of all children with ARTI complications, 1067 samples were infected by at least 1 respiratory virus accounting implying 51.7% of all enrolled cases. The distributions and proportions of each HRV species over the calendar months and years compared to other respiratory viruses were summarized in Fig 19. Based on the sequencing result and NJ

phylogenetic relationship of the VP4/VP2 with the previously defined prototypes, HRV were detected in 285 specimens and these HRV variants belonged to 57 different serotypes/ types of the 3 species including 28 HRV-C types, 21 serotypes and 8 serotypes of HRV-A and HRV-B, respectively (Table 3). This study identified a high proportion of HRV-C infections in children accounting for 49.1% followed by 28.4% HRV-A and 22.5% HRV-B.

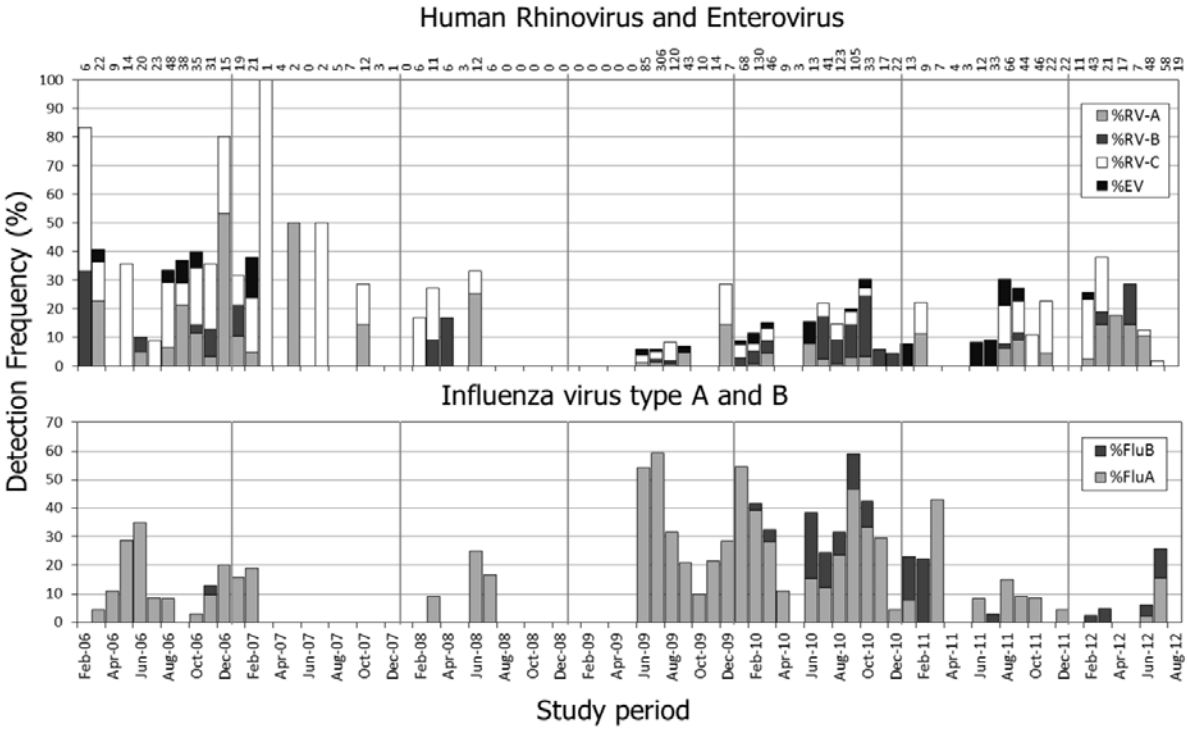


Figure 19: Distribution of monthly activity and proportion of the respiratory viruses between 2006 and 2012. The detection of respiratory virus including each species of HRV, HEV, Flu-A and Flu-B in each study month was plotted over the study period. The total numbers of respiratory specimens in each month are shown above in the bar graph.

The results from phylogenetic analysis and sequence identity matrix indicated that HRV-C was the major species identified in both of LRTI and URTI. This new virus species showed the most genetic variability compared inter and intraspecies. Wheezing and asthma exacerbation were the common clinical presentations of HRV-C positive LRTI cases. Most of HRV-C variants identified in Thailand were different from the references reported in the GenBank database and represent >30% nucleotide sequence divergences compered to inter-species. In contrast, HRV-A and HRV-B variants clustered with the previous known references reported from other countries. These findings highlighted the importance of

HRV as an aggravating factor for severe persistent respiratory disease in young children and reflected high genetic diversity of the virus disseminated in a short period of time.

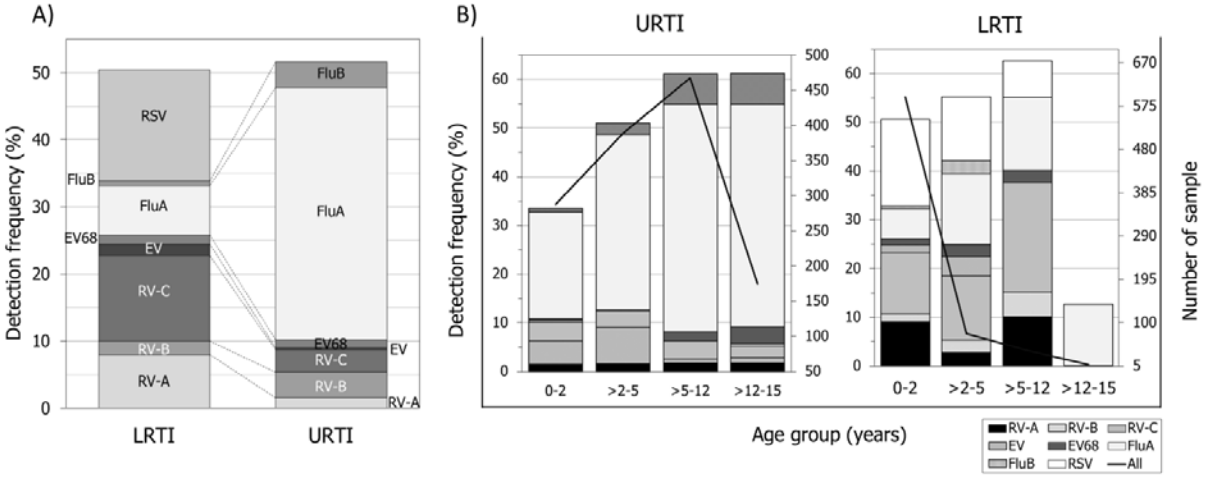


Figure 20: (A) Detection frequency of HRV and other viruses among the subject with different clinical severity and (B) distribution of the respiratory viruses with regard to the specific subjects' age.

Totally 35.9% and 64.1% were respiratory virus annual mean positive rate for LRTI and URTI, respectively. HRV annual prevalence was found in 13.8% of all ARTI cases and 8.8% of URTI subjects and showed the highest mean prevalence among LRTI subjects (22.7%) (Fig 20 and Table 3). HRV was the most respiratory virus detected in LRTI subjects followed by RSV (16.3%) and Flu (8%; 92% of Flu-A and 8% of Flu-B). The frequency of HRV co-infection with other respiratory viruses was found as the following; (1) 29.1% of URTIs were co-infected with Flu comprising 64.7% (n=22) pH1N1/2009, 29.4% (n=10) other subtype of Flu-A and 5.9% (n=2) Flu-B; (2) 8.8% and 4.1% of LRTIs were co-detected with RSV and Flu-A, respectively. The median age of enrolled HRV-associated LRTI and URTI subjects was 1 yrs (1 d to 11 yrs) and 4 yrs (1 m to 14 yrs), respectively. HRV activity in both of LRTI and URTI patients aged ≤5 yrs were statistical significance higher than other age groups (22.6% of LRTIs and 11.4% of URTIs). The result among children with ARTI complications showed that at least 1007 ARTIs were tested negative for HRV, Flu-A and Flu-B. Of these, 31.6% (n = 318) were LRTI subjects that tested negative for HRV, Flu-A, Flu-B and RSV type A and B and at least 34% (n = 108) of these samples were also tested negative for other common respiratory viruses including AdeV, Bocavirus, WU and KI polyomavirus, Paraflu, HEV, and HMPV.

Table 3: Proportion of HRV and other pathogens co-detected in the study population

HRV infection	LRTI (748)	URTI (1335)	ARTI (2083)
All HRV cases	22.7% (170)	8.8% (117)	13.8% (287)
HRV-A	35.3% (60)	17.9% (21)	28.2% (81)
HRV-B	8.8% (15)	43.6% (51)	23% (66)
HRV-C	55.9% (95)	38.5% (45)	48.8% (140)
Co-infection with Flu	4.1% (7)	29.1% (34)	14.3% (41)
With Flu-A	4.1% (7)	94.1% (32)	13.6% (39)
With Flu-B	0	5.9% (2)	0.7% (2)
Co-infection with RSV	8.8% (15)	-	-

Detection of HEV68 in ARTI patients and its genetic characteristic

Twenty-five ARTI cases were confirmed as HEV68 infection indicating a prevalence of 1.2% in the entire study population. To investigate the relationship between HEV68 characterized in Thailand (HEV68-TH strains) and the previously defined strains, phylogenetic tree of the 5'UTR/VP2 (612 nt) and partial VP1 region (257 nt) and their sequence identity matrix (Fig 21 and Table 4) were constructed. The 5'UTR/VP2 region of HEV68-TH strains exhibited 86.3-97.1% nucleotide identities to each other. HEV68-TH strains shared 71-84.1% nucleotide identity with the first identified strain 'Fermon' (AY426531) and 67.7-93.7% with those of strains identified from other countries. All HEV68 infected cases were confirmed by amplification of the VP1 gene. Despite performing semi-nested PCR and nested-PCR amplifications yielding a 2nd PCR product of ~850 nt, 450 nt or 250 nt, only 11/25 HEV68-positive specimens could be amplified. Fig 21(B) shows the phylogenetic tree of the capsid VP1 encoding gene. Comparisons of the nucleotide sequence of VP1 revealed 84.4-100% identity among HEV68-TH strains and 81.7-92.2% identity between TH-strains and the primary strains. The TH-strains shared 85.6-99.8% nucleotide identity with the HEV68 strains currently circulating or identified between 2008 and 2010. Results from HRV-HEV specific PCR amplification and nucleotide sequencing also revealed that 15 specimens had other HEV infections (Fig 21(A)). Of these, 1 specimen was classified as PV Sabin-2 with similar proportions of PV Sabin-3 (vaccine strains confirmed), CV-A9, Echo9, Echo6, and HEV109 whereas CV-B5 and CV-B2 were identified in 2 samples each. Five samples were positive for CV-A24. The overall frequency of detection was 0.8%.

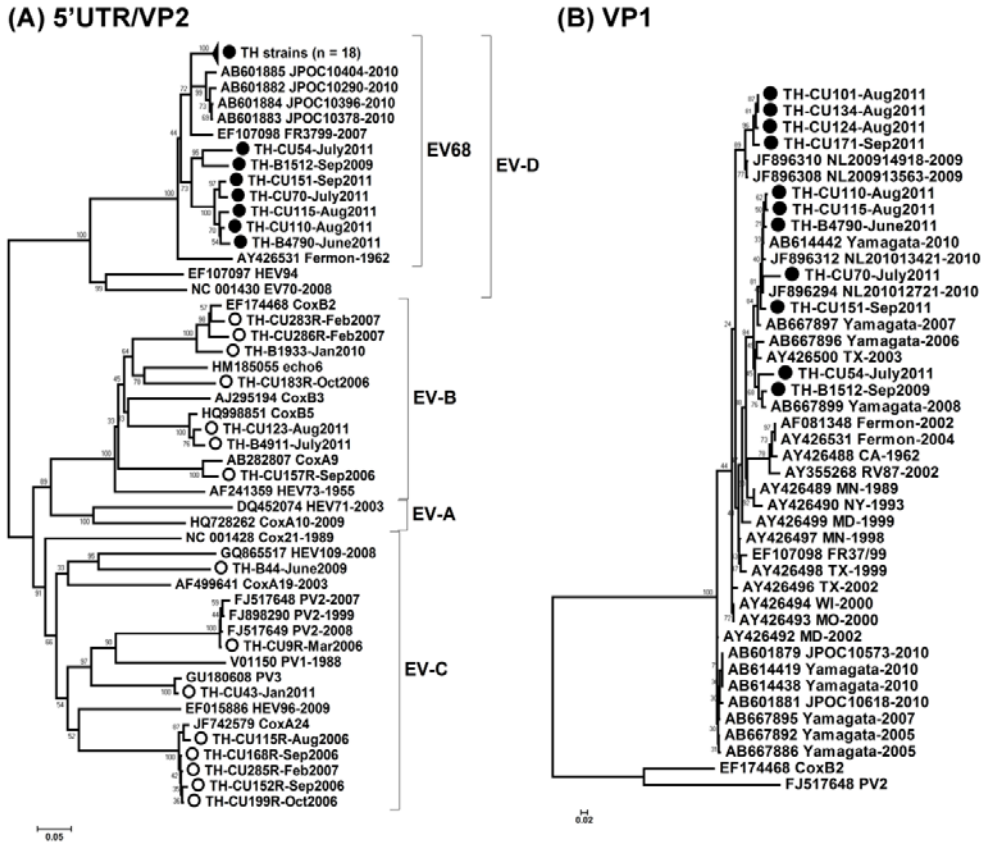


Figure 21: Phylogenetic relationship for HEV detected among children during 2006-2011. The NJ trees were constructed from nucleotide alignments of (A) the 5'UTR/VP2 and (B) partial VP1. The genetic distances were calculated according to the Kimura-parameter with 1000 bootstrapping. Scale bar indicates number of nucleotide substitutions per site.

Table 4: Nucleotide identity matrix obtained for the alignment of 5'UTR/VP2 of HEV68 identified from Thailand and reference strains and other HEV-D members.

Sequence	CU134	CU54	B2054	B2060	B1512	B196	HEV94	JPOC	FR3799	Fermon
CU54	86.2									
B2054	99.6	85.8								
B2060	99.8	86.0	99.4							
B1512	88.0	92.3	87.7	87.9						
B196	99.1	85.3	98.7	98.9	87.5					
HEV94	68.6	70.7	68.6	68.4	69.6	67.9				
JPOC	93.5	86.9	93.2	93.4	88.7	93.0	67.7			
FR3799	93.9	89.7	93.5	93.7	91.8	93.0	68.6	94.6		
Fermon	82.9	82.8	82.6	82.8	84.1	82.4	71.0	82.9	82.9	
HEV70	71.8	70.8	71.8	71.6	70.1	70.9	75.2	69.1	69.6	70.7

The GenBank accession numbers of the previously published sequences are as follows: HEV94, EF107097; HEV70, NC_001430; JPOC, AB601884; FR3799, EF107098; Fermon, AY426531.

Table 5: ARTI cases infected by HEV68 in Thailand from June 2009 to November 2011.

Group	Mild ARTI	Moderate ARTI	Severe ARTI	HEV68 cases	Average age
Infant (n=709)	1 (0.1%)	5 (0.9%)	1 (6.3%)	7 (1%)	1.5±0.7
Pre-school (n=420)	0	2 (0.3%)	0	2 (0.5%)	4
Primary-school (n=480)	9 (0.7%)	1 (0.2%)	0	10 (2.1%)	8.6±2.0
Secondary-school (n=175)	6 (0.5%)	0	0	6 (3.4%)	14.2±0.8
Total	16 (1.3%)	8 (1.4%)	1 (6.3%)	25	

Clinical manifestations of HEV68 infected patients

HEV68 were detected as a sole pathogen in 16/25 cases (64%). Co-infections by other respiratory viruses were found in 9 cases (36%) including 1 with RSV type A, 3 with Flu-B, and the remaining 5 patients were co-infected with pH1N1/2009. Age specifics of children with ARTI complications were 1% infant (7 cases; 1 outpatient and 6 hospitalized patients), 2% pre-school (2 cases; all were hospitalized cases), 2.1% primary-school (10 cases; 9 outpatients and 1 hospitalized patient), and 3.4% secondary school children (6 cases; all were outpatients) (Table 5). The average age of HEV68-associated ARTI cases was statistically higher than that of the non-infected group (7.6±5.0 yrs. vs 5.0±4.4 yrs.; p = 0.04). Without co-infection by other pathogens, the average age of HEV68-associated ARTIs was 5.8±4.4 yrs. The majority of HEV68 cases were primary school (40% of HEV68 and 2.1% of primary school cases) and secondary-school children (24% of HEV68 and 3.4% of secondary-school cases). Demographic data and clinical characteristics of these 25 HEV68-positive children are summarized in Table 6.

Of HEV68-positive children, 64% were children aged >5 yrs. (p = 0.016). Female-to-male ratio of HEV68 infected patients was 1.5:1. As for the contribution of the virus to disease severity, the majority of positive cases had moderate severity (32% (8/25)). One patient presented with severe clinical characteristics and required admission to the ICU. HEV68-associated ARTI represented 6.3% (1/16) of all severe cases. None of the patients died. During the study period, hospitalized patients with ARTI were more likely to be infected with HEV68 compared to outpatients (1.5% (9/597) vs 1.3% (16/1213)) without statistical significance support. Summary of discharge diagnoses of HEV68-confirmed cases included viral pneumonia (n = 8), asthma (n = 1), and ILI (n = 16). Clinical manifestations of the patients with HEV68 infections are shown in Table 7. Supplemental O_2 and medical

ventilation were required in all HEV68-hospitalized cases. The most common clinical characteristics of these patients were fever, cough and dyspnea (77.8%), and wheezing (66.7%). Hospitalization lasted a median of 5 d (3-16 d). Four patients with HEV68 infections had no medical history while 3 had underlying disease such as gastro-esophageal reflux, congenital heart disease, and autism and 2 had a history of asthma. One of these had RSV-A co-infection. However, there was no significant difference in disease severity between the case with and without RSV co-infection.

Table 6: Clinical characteristics and demographic data of the 25 cases of HEV68 infection.

Strain	Sample	Date	Age	Sex	Co-infection	Diagnosis	5'UTR/VP2	VP1
TH-B196	NS	30/6/2009	13	M	pH1N1/2009	ILI	JQ411799	N/A
TH-B211	NS	1/7/2009	14	F	pH1N1/2009	ILI	JQ411798	N/A
TH-B323	NS	4/7/2009	11	F	pH1N1/2009	ILI	JQ411797	N/A
TH-B521	NS	9/7/2009	10	M	pH1N1/2009	ILI	JQ411796	N/A
TH-B1512	NS	2/9/2009	15	F	-	ILI	JQ411794	JQ411802
TH-B2054	NS	9/2/2010	12	M	pH1N1/2009	ILI	JQ411791	N/A
TH-B2060	NS	10/2/2010	8	F	-	ILI	JQ411792	N/A
TH-B2114	NS	16/2/2010	9	M	-	ILI	JQ411789	N/A
TH-B2192	NS	24/2/2010	9	F	-	ILI	JQ411793	N/A
TH-B2213	NS	26/2/2010	8	F	-	ILI	JQ411790	N/A
TH-B2303	NS	12/3/2010	14	M	-	ILI	JQ411786	N/A
TH-B2340	NS	1/4/2010	6	M	-	ILI	JQ411788	N/A
TH-B2370	NS	2/6/2010	14	M	Flu-B	ILI	JQ411787	N/A
TH-B3611	NS	15/9/2010	15	F	Flu-B	ILI	JQ411781	N/A
TH-B4105	NS	5/10/2010	6	F	Flu-B	ILI	JQ411782	N/A
TH-B4790	NS	27/6/2011	1	F	-	ILI	JQ411785	JQ411809
TH-CU54	NP	8/7/2011	2	F	-	VP	JQ411783	JQ411805
TH-CU70	NP	25/7/2011	1	F	-	VP	JQ411784	JQ411801
TH-CU101	NP	15/8/2011	2	F	-	VP	JQ411776	JQ411804
TH-CU110	NP	18/8/2011	2	F	RSV-A	VP	JQ411777	JQ411810
TH-CU115	NP	22/8/2011	4	F	-	VP	JQ411778	JQ411811
TH-CU124	NP	26/8/2011	7	F	-	VP	JQ411779	JQ411807
TH-CU134	NP	31/8/2011	2	M	-	VP	JQ411775	JQ411803
TH-CU151	NP	12/9/2011	4	M	-	VP	JQ411780	JQ411806
TH-CU171	NP	23/9/2011	7m	M	-	VP	JQ411795	JQ411808

pH1N1/2009, pandemic influenza A virus subtype H1N1/2009; Flu-B, Influenza B virus; RSV, Respiratory syncytial virus; ILI, influenza like illness; VP, Viral pneumonia

Of HEV68-positive children, 64% were children aged >5 yrs. (p = 0.016). Female-to-male ratio of HEV68 infected patients was 1.5:1. As for the contribution of the virus to disease severity, the majority of positive cases had moderate severity (32% (8/25)). One patient presented with severe clinical characteristics and required admission to the ICU.

HEV68-associated ARTI represented 6.3% (1/16) of all severe cases. None of the patients died. During the study period, hospitalized patients with ARTI were more likely to be infected with HEV68 compared to outpatients (1.5% (9/597) vs 1.3% (16/1213)) but this difference did not show any statistical significance. Summary of discharge diagnoses of HEV68-confirmed cases included viral pneumonia (n = 8), asthma (n = 1), and ILI (n = 16). Clinical manifestations of the patients with HEV68 infections are shown in Table 7. Supplemental O_2 and medical ventilation were required in all HEV68-hospitalized cases. The most common clinical characteristics of these patients were fever, cough and dyspnea (77.8%), and wheezing (66.7%). Hospitalization lasted a median of 5 d (3-16 d). Four patients with HEV68 infections had no medical history while 3 had underlying diseases including gastro-esophageal reflux, congenital heart disease, and autism and 2 had asthma. One of these had RSV-A co-infection. However, there was no significant difference in disease severity between the case with and without RSV co-infection.

Table 7: Clinical manifestations of the 9 patients hospitalized with HEV68 infection.

Strain	LT	Underlying disease	Symptoms	O_2 therapy
TH-CU54	5	No	Fever, cough, dyspnea, wheezing	Yes
TH-CU70	3	No	Fever, cough, dyspnea, wheezing	Yes
TH-CU101	5	Autism	Fever, cough, vomiting	Yes
TH-CU110	4	No	Fever, cough, vomiting, diarrhea, dyspnea, wheezing	No
TH-CU115[a]	4	No	Tachypnea, wheezing, runny nose	Yes
TH-CU124[a]	7	No	Fever, cough, dyspnea, wheezing	Yes
TH-CU134	8	GER	Fever, cough, dyspnea, wheezing	Yes
TH-CU151	3	No	Fever, cough, runny nose, dyspnea, wheezing	Yes
TH-CU171[b]	16	CHD	cough, dyspnea, upper airway obstruction	Yes

a = had history of asthma/ b = admitted to the intensive care unit/ All of these HEV68 infected patients required nebulizer/ LT, Length of hospitalization (days); GRE, Gastro-esophageal reflux; CHD, Congenital heart disease

Seasonality of HRV and the outbreak of HRV69 in 2010

Over the 7-year study, ARTI caused by HRV infections were distributed throughout the year amid co-circulation of different species and variants, which were the common characteristics of HRV. Despite HRV showed no specific seasonality pattern, the virus was more obvious increased in rainy and early winter months except the year 2012 which the virus activity was observed in summer though rainy season. As for example, HRV-A and

HRV-C could be detected throughout the year in 2006 with statistically predominance during the rainy and early winter seasons (p<0.05). In 2010, the majority of HRV cases were detected in summer (17.7%) and mainly in the rainy seasons (72.2%) with a large proportion of HRV-B detection in September and October 2010, accounting for 61.4% (35/57) of all HRV cases during that time. Nucleotide sequences obtained from these HRV-B positive samples showed more than 84% nucleotide sequence identity compared with HRV-B serotype 69. This implied the outbreaks of HRV69 in Bangkok during the rainy 2010.

HRV activity correlates to meteorological factors in Bangkok, Thailand

The climate of Bangkok is tropical savanna or tropical wet and dry climate with daytime temperatures reaching the mid-30s^{o}C throughout the year. Based on the observations of the Thai meteorology department, the wettest period of the year is August to September and April is the hottest month. Average values, minimum, maximum, and median of each meteorological factor collected at Bangkok metropolis station (standard code 455201, located at latitude 13.43.35°N and longitude 100.33.36°E) during the study period are summarized in Table 8. To determine whether meteorological factors have an impact on or are associated with respiratory virus epidemics, 2-tailed Spearman's correlation coefficient analyses were conducted. Spearman's correlation coefficient showed significantly correlation between HRV activity and relative humidity. Moreover, this factors also showed the association with the increase number of LRTI in Bangkok (r_s = 0.211, p = 0.021). In contrast, the number of observed LRTI case appears to have inverse correlation with the weather temperature in Bangkok (Table 8). This study further analyzed the association of HRV activity and the respiratory disease severity with climatic factors during the study period. The results showed that the frequency of HRV-associated URTIs detection showed statistically correlated with average rainfall (r_s= 0.218, p = 0.049), relative humidity (r_s = 0.224, p = 0.043) and number of rain day (r_s = 0.220, p = 0.047) calculated per 2 weeks while one of climatic showed significantly correlation with HRV activity in LRTIs. Furthermore, an increase number of HRV-B activity in URTI subjects was the only one HRV species directly correlated with an increase of average relative humidity. In comparison with HRV activity, an increase of the number of LRTIs with RSV-infection showed more correlation with climatic factor than HRV. It shows direct correlations with average relative humidity, number of rain day and rainfall and inverse correlation with weather temperature.

Table 8: The assessment of correlation between the meteorological factors and the number of ARTI cases and the infections caused by HRV and other respiratory viruses

Meteorological factors	**Temperature (°C)**		**Relative Humidity (%)**		**Rainfall (mm)**		**Rain day (day)**	
Min, Max	25.36,32.56		55.57, 86.57		0, 132.9		0,14	
Median	29.05		73.61		0		5.5	
Mean±SD	29.03±1.20		72.44±6.64		6.95±16.37		5.56±4.06	
Correlation	r_s	***p***	r_s	***p***	r_s	***p***	r_s	***p***
No. of ARTI case	-0.050	0.545	0.236**	0.004	0.007	0.933	0.147	0.074
No. of LRTI case	-0.044	0.634	0.211*	0.021	0.145	0.114	0.169	0.065
HRV infection	0.032	0.732	0.062	0.498	-0.026	0.776	-0.002	0.979
HEV infection	-0.145	0.115	0.182*	0.047	0.162	0.077	0.215*	0.018
RSV infection	-0.049	0.595	0.263**	0.004	0.191*	0.037	0.269**	0.003
No. of URTI case	0.009	0.935	0.150	0.178	0.090	0.420	0.127	0.255
HRV infection	-0.015	0.897	0.224*	0.043	0.218*	0.049	0.220*	0.047
Flu-B infection	-0.029	0.797	0.289**	0.008	0.218*	0.049	0.257*	0.020

Table 9: Correlation of meteorological factors with respiratory virus activity in ARTI.

Correlation analysis		**ARTI**	**ARTI with different respiratory virus infection**								
Climate factors	**Correla.**	**All**	**HRV**	**HRV A**	**HRV B**	**HRV C**	**HEV**	**HEV 68**	**Flu**	**Flu A**	**Flu B**
Rain fall	r_s	.007	.170*	.107	.174*	.086	.037	-.070	-.037	-.046	.076
	Sig.	.933	.038	.197	.034	.298	.657	.397	.652	.575	.357
Temp.	r_s	-.050	-.038	-.049	-.030	-.009	-.062	.005	-.036	-.031	-.052
	Sig.	.545	.642	.553	.714	.914	.456	.952	.665	.708	.529
RH	r_s	.236**	.209*	.023	.244**	.157	.205*	.199*	.162*	.137	.266**
	Sig.	.004	.011	.786	.003	.056	.012	.016	.049	.097	.001
Number of rain day	r_s	.147	.131	.061	.150	.069	.159	.160	.103	.083	.203*
	Sig.	.074	.111	.465	.069	.403	.053	.052	.213	.314	.013

Table 10: Correlation of meteorological factors with respiratory virus activity in URTI.

Correlation analysis		**URTI**	**URTI with different respiratory virus infection**								
Climate factors	**Correla.**	**All**	**HRV**	**HRV A**	**HRV B**	**HRV C**	**HEV**	**HEV 68**	**Flu**	**Flu A**	**Flu B**
Temp.	r_s	.009	-.015	.034	-.035	-.005	.117	.116	-.004	-.001	-.029
	Sig.	.935	.897	.760	.753	.966	.294	.299	.973	.991	.797
RH	r_s	.150	.224*	.059	.272*	.099	.093	.069	.134	.111	.289**
	Sig.	.178	.043	.598	.014	.375	.407	.538	.229	.321	.008
Number of rain day	r_s	.127	.220*	.103	.200	.160	.014	.010	.116	.095	.257*
	Sig.	.255	.047	.358	.071	.151	.903	.932	.298	.394	.020
Rain fall	r_s	.090	.218*	.054	.234*	.138	-.023	-.038	.079	.061	.218*
	Sig.	.420	.049	.633	.034	.218	.841	.732	.482	.589	.049

r_s = Pearson correlation coefficient/ p = P-value/ * correlation is significant at the 0.05 level / **correlation is significant at the 0.01 level

Table 11: Correlation of meteorological factors with respiratory virus activity in LRTI.

Correlation analysis		LRTI	LRTI with different respiratory virus infection									
Climate factors	**Correla.**	**All**	**HRV**	**HRV A**	**HRV B**	**HRV C**	**HEV**	**HEV 68**	**Flu**	**Flu A**	**Flu B**	**RSV**
Temp.	r_s	-.044	.032	-.030	.083	.051	-.145	-.087	-.126	-.126	-.007	-.049
	Sig.	.634	.732	.742	.368	.577	.115	.347	.170	.171	.936	.595
RH	r_s	.211*	.062	-.011	-.016	.113	.182*	.218*	.076	.052	.118	.263**
	Sig.	.021	.498	.908	.864	.220	.047	.017	.408	.576	.200	.004
Number of rain day	r_s	.169	-.002	.026	-.032	-.015	.215*	.275**	.124	.109	.072	.269**
	Sig.	.065	.979	.781	.731	.869	.018	.002	.179	.234	.436	.003
Rain fall	r_s	.145	-.026	-.024	-.033	-.014	.162	.174	.083	.076	.040	.191*
	Sig.	.114	.776	.796	.722	.877	.077	.058	.365	.411	.666	.037

r_s = Pearson correlation coefficient/ p = P-value/ * correlation is significant at the 0.05 level / **correlation is significant at the 0.01 level

Table 12: Linear regression analysis for LRTI activity and HRV-associated hospitalizations of pediatric patients based on relative humidity.

Factor (s)		Regression analysis					
Dependent	Independent	β[a]	*t*	*p*	95%CI[b]	R^2	Equation
LRTI	RH[c]	0.211	2.342	0.021	0.032 to 0.388	4.4	LRTI = 0.210RH-8.956
HRV	RH[c]	0.224	2.058	0.043	0.003 to 0.187	5	HRV = 0.095RH-5.595

[a] Standardized β-coefficient/ [b] 95% confidence interval/ [c] Relative humidity (%)

To gather the preliminary information of the relationship between the number of LRTI and HRV-associated URTI cases and the climatic factors determined above as well as establish the distribution pattern in each calendar month, the stepwise fitted method was used for significant covariation analyses. Simple linear regression equations were formulated using the coefficient between correlated factors created by multiple regression analysis. The R^2 of the LRTI-fitted model was 4.4 with 0.211 of standardized β-coefficient. Model implied the linear relationship between the number of LRTI case and relative humidity and calendar months. By using the simple linear regression equation, the percentage of LRTI cases was calculated and compared to the percentage of observed LRTI cases per 2 week of 2006-2008 and 2010 through 2012. The annual oscillation and distribution pattern of predicted LRTI cases show in Fig 22. The correlation coefficient between predicted and observed LRTI cases was 0.211 with a p-value of 0.021. The R^2 of

the fitted model was 5 with 0.224 of standardized β-coefficient, indicating increasing HRV activity with increasing relative humidity. The correlation coefficient (r_s) between the 2 types of number of HRV-associated URTIs was 0.272 with a 0.014 p-value.

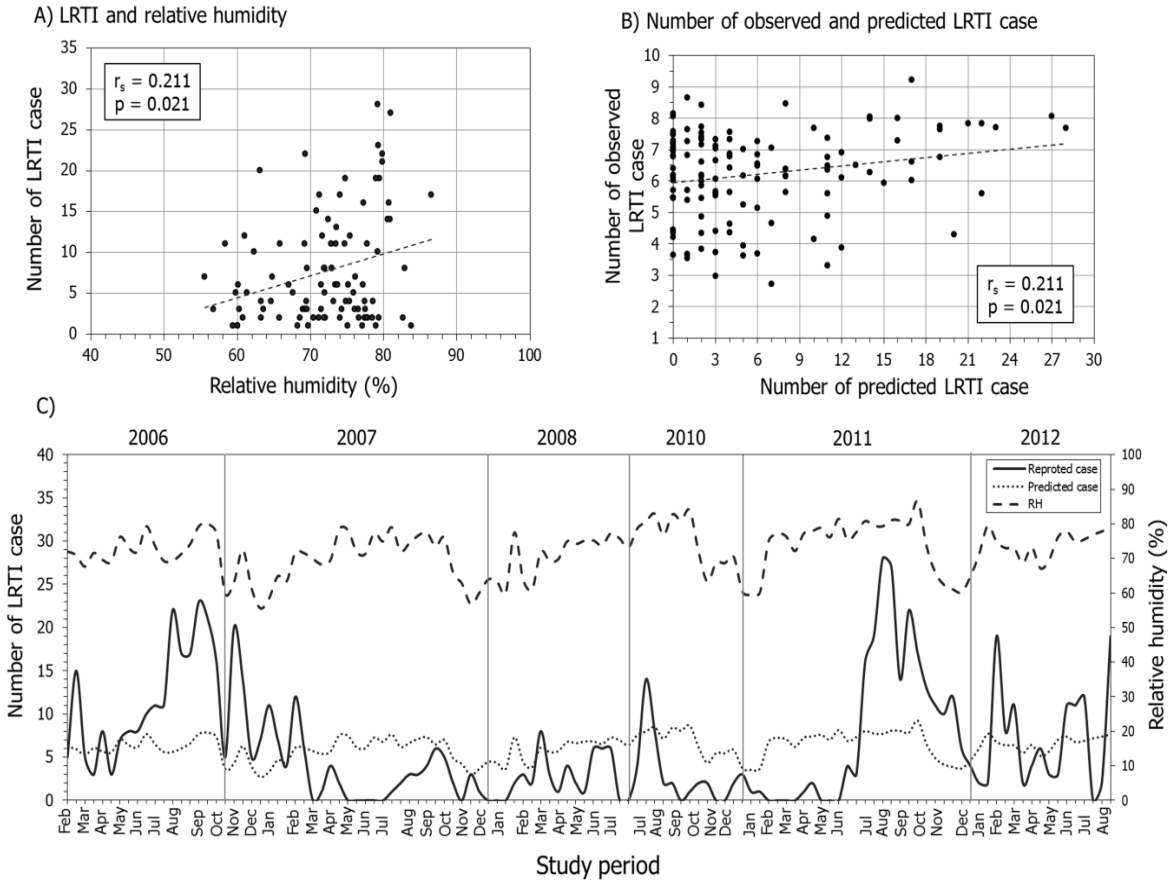


Figure 22: (A) Relationships between observed number of LRTI cases and average relative humidity per 2 weeks in Bangkok, Thailand. The (B) correlation and (C) annual oscillation between calculated and observed number of LRTI cases per 2 weeks during the study period. Predicted LRTI cases were calculated on the basis of linear regression analysis of an average relative humidity.

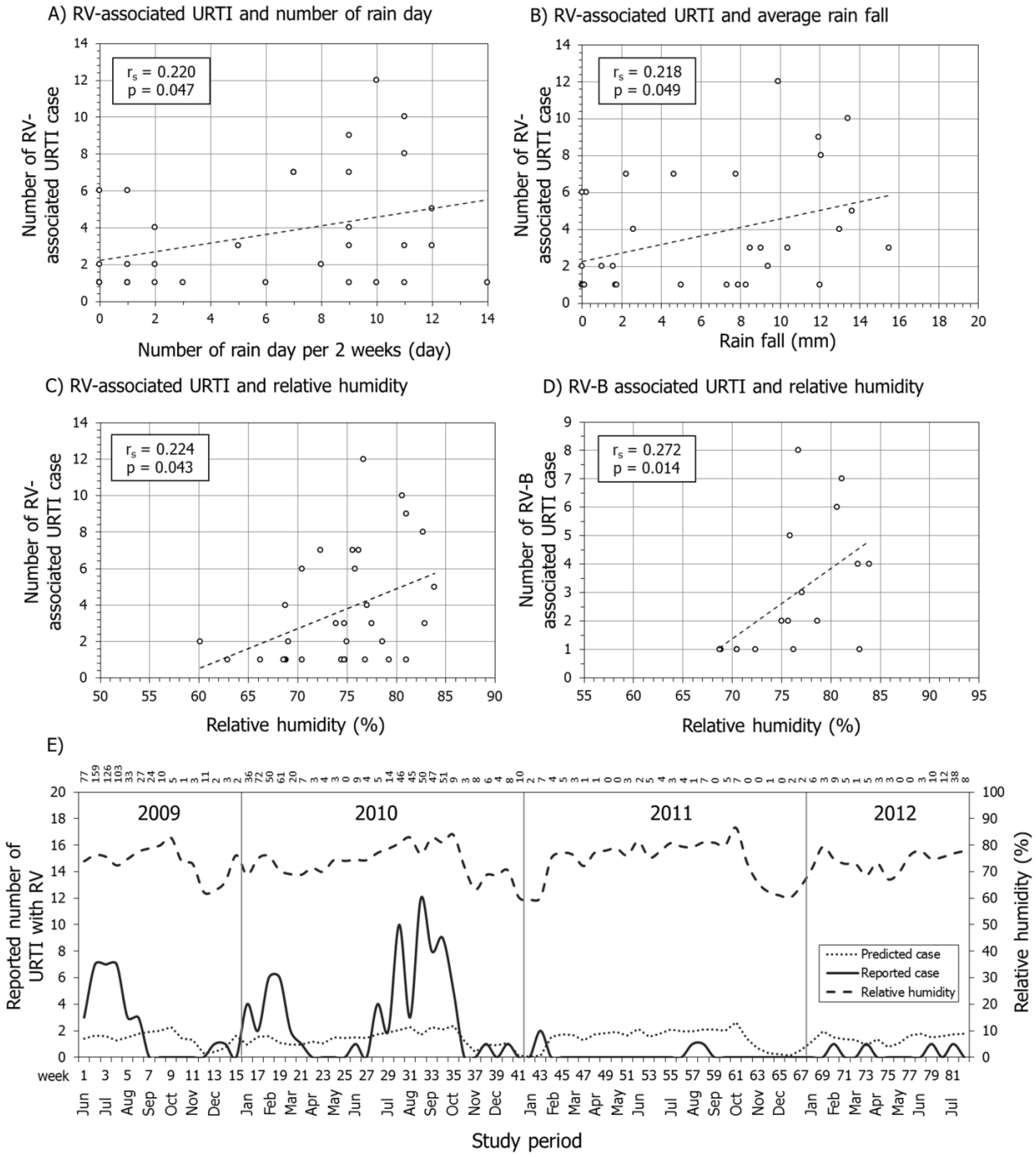


Figure 23: (A)-(D) Relationships between observed number of URTT cases with HRV infection and average relative humidity. The viral trends are daily 2 weeks moving averages. Spearman correlation coefficient and p-value are shown in each graph. (E) Comparison of epidemic curves of reported and calculated number of URTI-associated with HRV infection in Bangkok between 2009 and 2012, based on the predicted seasonal model.

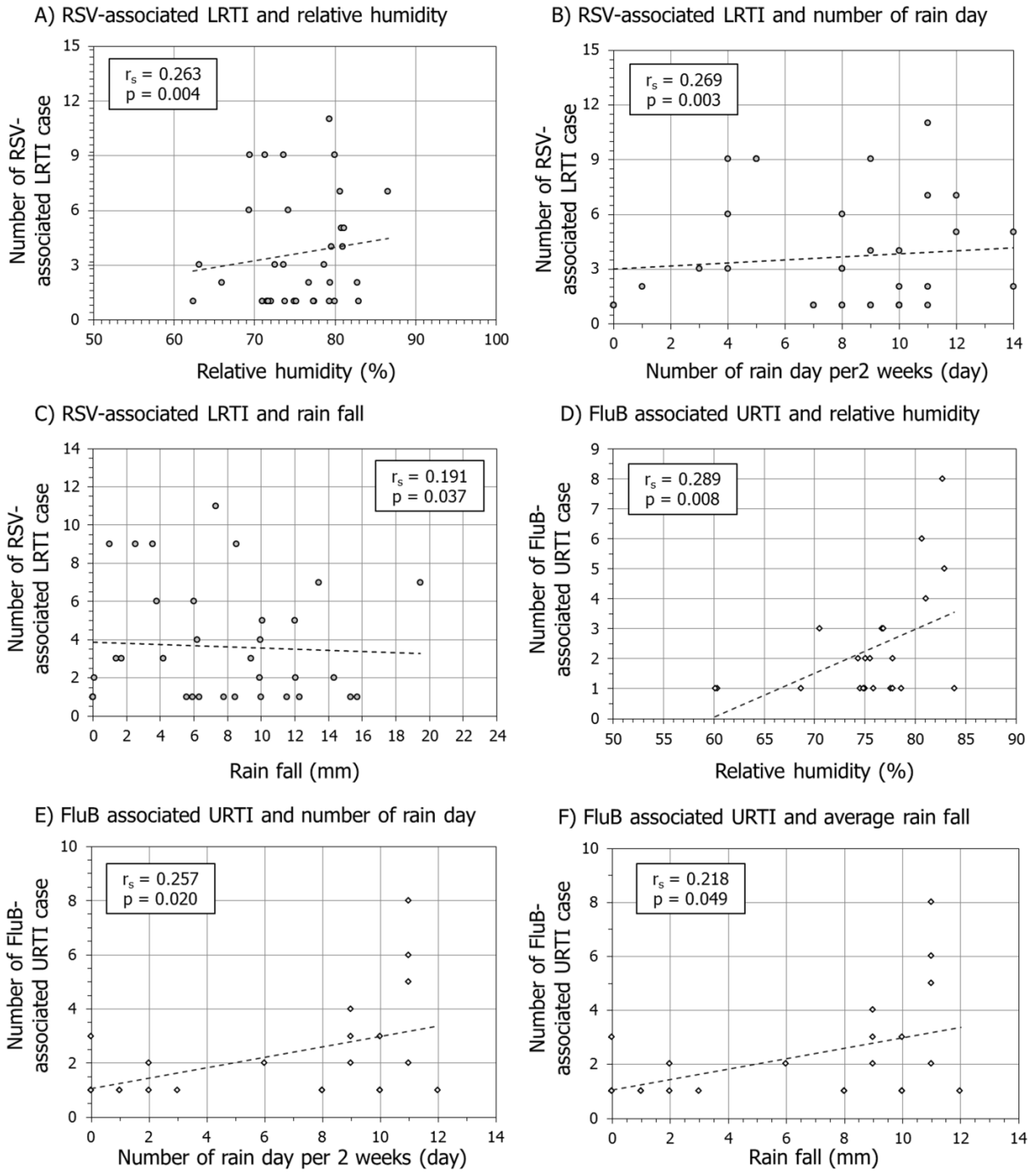


Figure 24: Relationship between meteorological parameters and reported numbers of respiratory illness (URTI or LRTI) case among Thai children during 2006 to 2012.

Seasonality of HEV68

During the first 2 years (Feb 2006-Jul 2008), there was no HEV68 infection in the sample population (n = 383). Fig 25 provides epidemiological profile of HEV68 infection among the patient samples identified in Thailand during 2009-2011. In 2009, 5 HEV68 cases were detected in Jun, Jul, and Sept (rainy season) with 0.9% annual prevalence (5/584; all were outpatients). Increased incidence of HEV68 infection associated with ARTI was found in 2010 and 2011. In 2010, the virus could be found during a 6-month period with 1.6% annual prevalence (10/611; all were outpatients). During 2011, HEV68 infections were investigated only during the rainy season with 4.3% annual prevalence (10/232; 1 outpatient and 9 hospitalized patients). Of all HEV68 infections detected, 8% were identified from children admitted to the hospital in summer, 72% during rainy season, and 20% in winter.

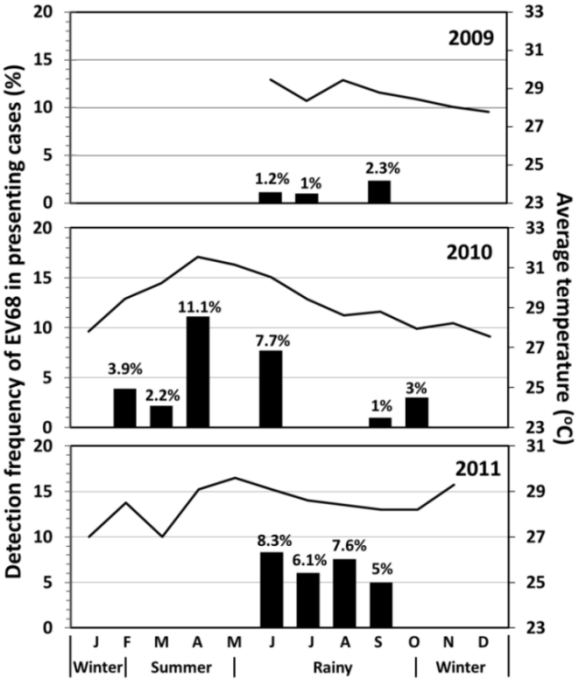


Fig 25: Seasonal distribution of HEV68 in Bangkok, Thailand combined from 3 years (2009-2011) during which HEV68 cases had been reported. Bar graph shows percentage of pediatric patients with HEV68 infection and line graphs indicate monthly average temperatures (^{o}C) in Bangkok.

No HEV68 case was reports in 2012. Among the meteorological factors, relative humidity was the only factor statistically correlated with the overall number of HRV-HEV infected patients particularly in 2011 (r_s = 0.61, p = 0.04). None of the meteorological factors correlated with the frequency of HEV68 detection in Thailand.

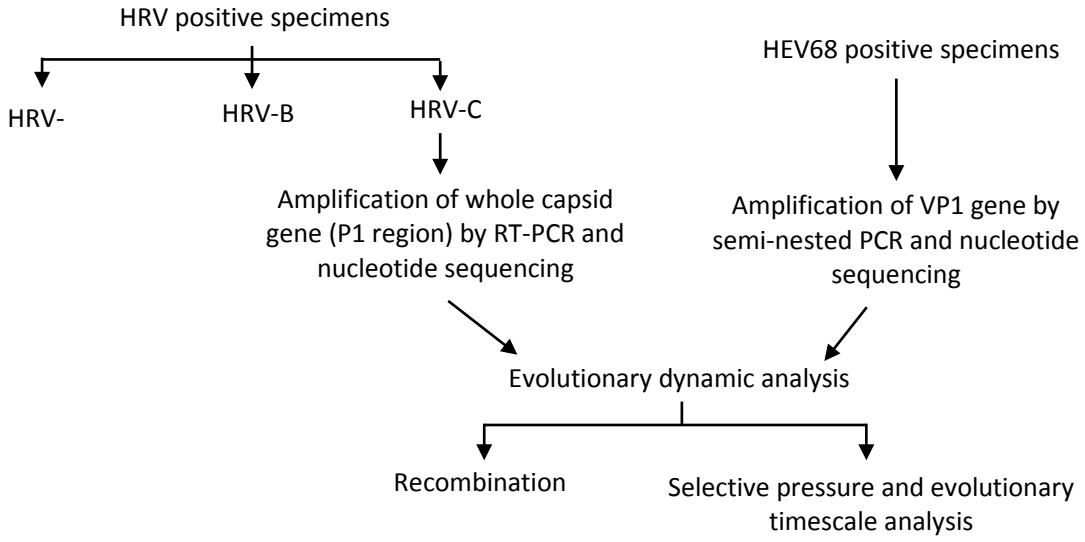
Part 2: Evolutionary dynamics of HRV and HEV68.

HRV and HEV are known for high genome plasticity as consequences of high mutation rates and recombination event. To further explore the genetic characteristics and evolutionary divergence of the high genetically diverse HRV, this study characterized the full-length coding sequence of the 6 representative HRV strains circulating in Thailand and comparatively analyzed recombination in each of the HRV species. Furthermore, with the recent increase in the number of partial capsid sequences and complete genome sequences enables a more detailed analysis of the HRV and HEV68 evolutionary characteristics. This study genetically characterized molecular timescale and evolutionary relationships of HRV circulating lineages by using the global published sequences of 5'UTR, VP4/2, VP1 and/ or structural P1 regions in order to elucidate many aspects of their evolutionary history and genetic complexity and to determine how evolution of this virus might have been shaped by its population dynamics. The secondary objective of this study was therefore to better understand the evolutionary history of HRV especially the recently new HRV-C and HEV68 circulating lineages and to determine how evolution of this virus might have been shaped by its population dynamics.

Research questions

1. What is the common ancestor of HRV, particularly HRV-C, and HEV68 and when did the diversification occur?
2. Which kinds of evolution forces drive the genetic heterogeneity in HRV-C and EV68?

Conceptual framework



Objectives

1. To trace back the transmission chains of HRV and HEV68 during the course of an epidemic
2. To gain insight into the evolutionary processes that creates the genetic diversity and molecular epidemic among HRV-C and HEV68 circulating lineages

Expected benefits and applications

1. Knowledge on the role of diversification factors acting on the novel or re-emerging virus evolutionary processes.
2. The strategies can be applied in evaluation of the evolutionary analysis in other viruses.

Materials and Methods

PCR amplification and nucleotide sequencing

Primer sets for HRV entire coding sequence amplifications were designed based on each species' specific nucleotide sequence available at the GenBank database (Appendix D). The sequences of the genome termini were arrived at by a specific PCR technique developed from a modified 3′RACE [28]. All purified PCR products were bi-directionally sequenced with the 2 primers used in the second round of semi-nested PCR provided by First BASE.

Complete coding sequence analyses

Sequences were prepared and aligned using ClustalW implemented in the BioEdit. Pairwise Sequence similarity plot was calculated and depicted using SimPlot [135] with Jukes-Cantor parameter, window size of 400 bp and a step size of 20 bp. To examine the picornaviral protease cleavage sites ($2A^{pro}$, $3C^{pro}$, autocatalytic sites), sequences were sought using the Net-PicoRNA 1.0 server [136]. Consensus *cr*e sequences of the selected alignment regions were evaluated using the RNAalifold and MFold server [137].

Phylogenetic compatibility matrix

Phylogenetic compatibility matrix (PCM) analysis is a computational method used to investigate the phylogenetic relationship of the sequences to be analyzed. The PCM plot of nucleotide sequence alignment in intra- and inter-HRV species was constructed by using the program TreeOrderScan in the Simmonic 2007 version 1.6 [138]. All published HRV reference nucleotide sequences of each species including 75 HRV-A, 25 HRV-B, 9 HRV-C, and 6 identified strains were aligned and computed separately between and

within species using the programs SEQBOOT, DNADIST, NEIGHBOR-JOINING and PHYLIP with the following program setting: 250 bp fragment length, 100-bp increments, 100 fold resampling with 70% bootstrap threshold value that subsequently generated 65 aligned fragments of HRV-A and HRV-B while HRV-C was generated from 64 overlapping fragments.

Recombination analysis

Potential recombination events within the coding regions were assessed using phylogenetic analysis based on the various viral genome parts with high recombination rate. To confirm an accurate recombination event, the complete coding sequences were analyzed in comparison with all known reference sequences by using the Recombination Detection Program 3Beta41 [139]. Manual Bootscanning was performed by using Jukes-Cantor algorithm and neighbor-joining method [28, 32, 106] with a parameter setting of 200 bp window size, 10 bp step size and 1,000 bootstrap replicates.

G+C content analysis

To analyze the G+C content of the full-length coding sequences of each HRV species, a total of 20 HRV-A, 25 HRV-B, and all HRV-C coding sequences available at the GenBank database were selected. Three representatives of each HEV species as well as 3 distinct PV were chosen from the database under the following GenBank accession numbers: HEV-A (DQ452074, AY421760, and AY421769), HEV-B (AF241359, AF081485, and AF029859), HEV-C (NC_001428, AF499640, and AF499635), HEV-D (NC_001430, EF107098, and DQ201177), and PV (V01150, X00595, and X00925). The %GC composition was directly compared within the viral reading frame and plotted with standard deviation by using online program including CpG ratio/ GC content (http://mwsross.bms.ed.ac.uk/public/cgi-bin/cpg.pl) and GC content/ GC skew diagrams (http://nbc11.biologie.unikl.de/framed/left/menu/auto/right/GC/) with a parameter setting of 500 bp sliding window and 10 bp increment size between successive windows.

Estimated rate of evolution and divergence time of the recent spread of HRV and HEV68

1. HRV 5′UTR, VP4/2 and VP1 dataset preparation

Datasets of the 5′UTR and VP4/VP2 sequences were constructed to perform the Bayesian phylogenetic investigations for estimating the divergence time and determining the most common recent ancestor (TMRCA) of the recently circulating HRV-C lineages. The

nucleotide datasets were established as follows: data set 1 was formed by including 390 nt-in-lengths of VP4/VP2 gene of available HRV-C strains (300 nucleotide sequences) with known collection date retrieved from GenBank database by November, 2012 and additional nucleotide sequences identified in the previous studies under accession numbers FJ435235-FJ435337, JN415562-JN415630 and KC412912-KC413018. Data set 2 and 3 were the nearly complete 5'UTR (420 nt, 126 sequences) sequences and VP1 gene (850 nt, 173 sequences) [10, 57]. The homologous sequences of other HRV comprising species A and B were included in this data set. Other HEV species were assigned as outgroup. Each sequence was labeled with its corresponding year of sampling or isolating.

2. HEV68-VP1 data set

Datasets of the VP1 gene were constructed to perform the Bayesian phylogenetic investigations for estimating the divergence time and determining TMRCA of the recently circulating HEV68 lineages. Nucleotide datasets of the VP1 gene were established as follows: dataset 1 was formed by including almost all available HEV68-VP1 sequences retrieved from GenBank database by October 31, 2011 and additional nucleotide sequences identified in this study (HEV68-TH strains). The homologous sequences of other HEV comprising HEV94 for species D (n = 2), CV-A10, HEV90 and HEV76 as a representative of species A (n = 4) were included in this data set. HEV species B and C were assigned as outgroup. Each isolate was labeled with its corresponding year of sampling or isolating. In addition, an alignment of data set 1 containing 122 sequences corresponding to the VP1 gene was constructed (723 nt). Dataset 2 included only HEV68-VP1 sequences. The total sequence number of this dataset was 106 sequences with the same length as data set 1.

Bayesian Markov chain Monte Carlo evolutionary analyses of HRV and HEV68

The rate of evolution, molecular clock phylogenies, and divergence time were estimated for 5'UTR and capsid VP4/2 gene sequences of HRV and VP1 of HEV68 using a Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in the BEAST (v1.6.2). To reduce excessive computational load, closely related sequences collected at the same laboratory and time point were manually removed without compromising the genetic heterogeneity of each alignment, resulting in a down-sampling of the HRV representative sequences. The best fit substitution model for each gene was analyzed by performing a maximum likelihood analysis using the Modeltest (v3.7). The best-fit clock

model suitable for molecular time scale analysis was demonstrated using an analysis of Bayesian model comparison. All analyses were carried out assuming a general time-reversible model of sequence evolution with a gamma-distributed among site rate variation (GTR+I+G) with four rate categories (γ_4). Alignments were performed by keeping the first and second codon positions in one partition and the third position in a separate partition as (1+2)+3 codon partitions. In order to accommodate rate variation at the third codon position, the relative rate parameter was separately estimated in each partition.

1. HRV best-fit substitution model and parameter for Bayesian MCMC analysis

Analyses were carried out with a Bayesian approach that does not assume a pre-specified model of demographic history, the Bayesian skyline plot (piecewise skyline model), and with methods that relax the assumption of a strict molecular clock for capsid VP4/2 and uncorrelated exponential molecular clock for 5'UTR. Parametric approaches for Bayesian analysis were optimized as following; operators were auto-optimized through an MCMC chain length involving 300 million and 500 million generations for 5'UTR and VP4/2, respectively. The MCMC procedure was sampled to obtain 10000 final trees. Convergence of the chains, mean estimated rate and 95% HPD interval evaluated for each operator were compiled by computational run over a sufficient time with inspection of the MCMC samples during 10% burn-in period using TRACER (v1.4). Maximum clade credibility trees were estimated using Tree-Annotator (v1.4.8) and annotated by using FigTree (v1.1.2). Topological support of the analyzed tree was assessed by posterior probability (pp) values.

2. HEV68 best-fit substitution model and parameter for Bayesian MCMC analysis

Relaxed lognormal molecular clocks were employed and followed by allowing substitution rate variations among branches on the trees. Molecular model specification was selected using Bayes Factors and used to quantitatively estimate the growth rate and demographic parameter. The dynamic among study populations was estimated by performing a Bayesian Skyline plot. The Bayesian MCMC chain lengths were 10 million generations with sampling every 10000 generations and discarding 10% of the chain as burn-in. Convergence of the chains was achieved by computational run over a sufficient time with inspection of the MCMC samples using TRACER. The resulting tree of each run was summarized using Tree Annotator and the maximum clade credibility tree was visualized with FigTree program.

Measurement of selective pressure

Codon-based models of evolution of coding sequence allowing for variable selection pressure among site in a maximum-likelihood framework were used to evaluate the selective pressure operating capsid VP4/2, VP1 and/ or P1. The ratio of non-synonymous substitution to synonymous substitution changes (ω = *dN/dS*) across the lineages on a codon by codon basis and the individual site specific selection pressure were measured using single likelihood ancestor counting (SLAC) and fixed-effects likelihood (FEL) model contained in the HYPHY accessed on Datamonkey website. The ω value of each codon was estimated based on neighbor-joining trees with the TrN93 substitution model. Selective pressure was defined as follows: $\omega = 1$ indicates neutral evolution, $\omega < 1$ indicates purifying or negative selection, and $\omega > 1$ indicates positive selection. The cutoff *p*-value for the FEL model was determined using chi-squared asymptotic test. The significance level for a positively or negatively selected site of SLAC and FEL analyses was estimated using two-tailed extend binomial test and accepted at the level of $p < 0.1$. The relative residue abundance within were depicted using WebLogo.

Results

Complete coding sequence analysis of HRV and the putative new strain, HRV-CU072

The entire coding sequences of the 6 additional HRV strains elucidated in this study have been submitted to the GenBank database and assigned accession numbers HQ123440-HQ123445. Nucleotide and deduced amino acid sequence analysis revealed considerably different phylogenetic clustering features of the strains HRV-CU072 (HQ123440) and HRV-CU211 (HQ123444) as showed in Fig 26.The strain HRV-CU072 displayed relatively low pairwise sequence identity compared with other HRV-C (66%).

To investigate the molecular characteristics of the putative new strain, this study performed comparative analysis of the HRV-CU072 complete coding sequence with all available HRV references and the representative of HEV. The HRV-CU072 coding sequence spanned 6,450 nt region rich in A and U bases and encoded a 2,149 aa polyprotein. It had a relatively small polyprotein gene due to a deletion in the major part of the antigen neutralization site covering the BC, DE, and HI loops of the VP1 and shared 50% and 45% amino acid sequence identity with HRV-A and HRV-B, respectively. Direct investigation of the VP1 gene revealed that HRV-CU072 shared only 64% nucleotide identity with the other members of HRV-C.

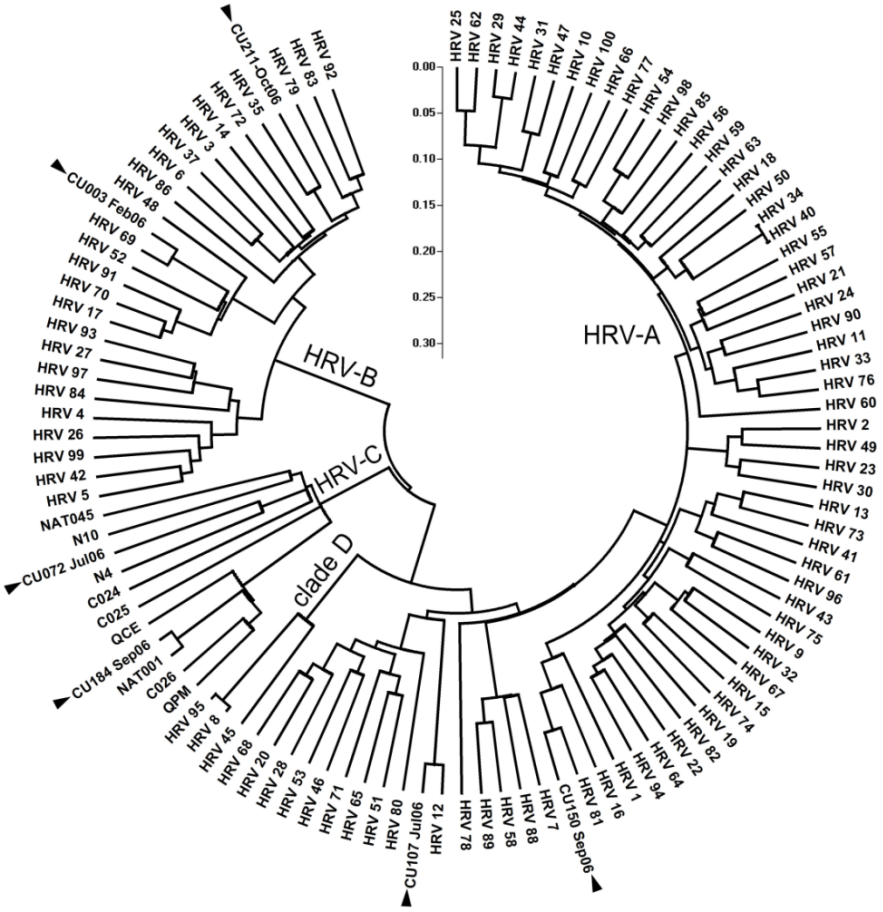


Figure 26: Phylogenetic analysis illustrating genetic relationships between HRV based on sequence alignment of 6 complete coding sequences amplified from this study (▲) compared with all known prototypes. Evolutionary distance was represented by the scale bar in the unit of nucleotide substitutions per site. The taxon name refers to number of specimen, admission month and year.

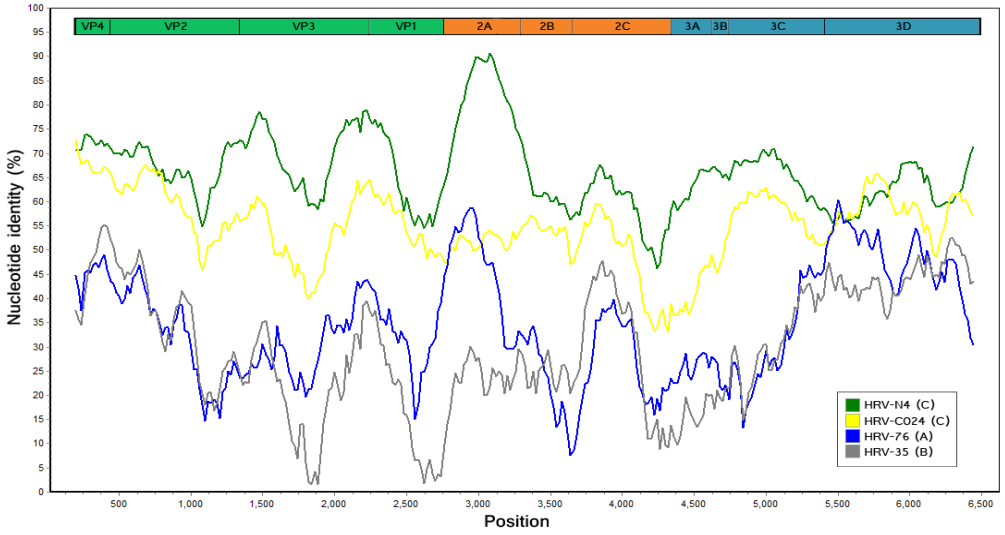


Figure 27: Complete coding sequence similarity plot illustrating pairwise sequence identity between HRV-CU072 compared with the most closely related strain N4 (green line) and other HRV members (HRV-C024; yellow line, HRV76; blue line, HRV35; gray line).

Table 13: Amino acid residues within viral-encoded protease cleavage sites of the HRV-CU072 polyprotein compared with putative sites of other HRV species.

Protein junction	CU072	HRV-C	HRV-A	HRV-B
VP4/ VP2	M/S	M/S	Q/S	N/S
VP2/ VP3	Q/G	Q/G	Q,E/G	Q/G
VP3/ VP1	N/D	Q/N	Q/N	E/G
VP1/ 2A	V/G	A,L/G	A,F,V,Y/G	Y/G
2A/2B	Q/G	Q/G	Q/G	Q/G
2B/2C	Q/S	Q/G,S	E,Q/S	Q/A,S
2C/3A	Q/G	Q/G	Q/G	Q/G
3A/3B	Q/G	Q/G	Q/G	Q/G
3B/3C	Q/G	Q/G	Q/G	Q/G
3C/3D	Q/G	Q/G	Q/G	Q/G

An estimated sequence variation was calculated using pair-wise nucleotide and deduced amino acid sequence alignment and indicated as a percentage of each individual viral protein.

An alignment of amino acid sequences was generated allowing for the 10 hypothetical cleavage sites of the CU072 polyprotein. In addition, half of all cleavage sites of the CU072 conserved amino acid residues were commonly found in HRV members while some cleavage site features, such as an identical M/S pair in the autocatalytic cleavage site between the structural proteins VP4 and VP2, were also found in CU072 and other HRV-C. The unique amino acid sequences of CU072's protease cleavage site were observed at the VP3/VP1 site as N/D residues while other HRV-C members utilized an alternative cleavage Q/N pair similar to HRV-As. However, amino acid polarity remained unchanged. Comparison of HRV-CU072's individual protein products with other HRV-C members showed that the VP4 protein was a highly conserved protein among other HRV and HEV species. Similar with other HRV-C members, CU072 displayed a *cis*-acting replication element (*cre*: R1NNNAAR2NNNNNNR3) as GCUUAAACAAAUUA located in the VP2 protein different from HRV-As and HRV-Bs where the cre structure is located in the 2A and 2C region, respectively. The G(P/A)Y(S/T)GxP motif within the 3B protein or VPg crucial for phosphodiester linkage formation between the VPg protein and 5´end of viral RNA was identified in CU072. Furthermore, at position 4 of this motif, almost all HRV-C displayed the unique Thr while only CU072, C025 (EF582386), N4 (GQ223227), and N10 (GQ223228) shared the conserved Ser in common with HRV-A and HRV-B.

To determine cell-specific receptor usage (ICAM-1 and LDLR), conserved motif and functional domain of the HRV-CU072 strain, the deduced amino acid sequences of protein

VP1 and carboxy-terminal VP3 were aligned. In total, 5 of 9 and 4 of 7 conserved residues corresponding to the ICAM-1 footprint of the HRV-A and HRV-B major group members, respectively, were found in the CU072. The fully conserved residue Gly1148 shared between the HRV-A major and minor group was also identified in the CU072 but the key residue Lys224 within the TEK motif located in the VP1 essential for HRV-virion and LDLR protein interaction was not found in CU072. An 8-10 amino acid insertion found in VP1 of CU072 represented some characteristics unique from other HRV members, such as a hydrophilic amino acid insertion in the GH loop. Furthermore, the HRV-CU072 strain might be resistant to pleconaril due to amino acid substitutions in the 2 positions (152 and 191) crucial for identifying naturally resistant serotypes located in the drug binding pocket identified as Y52F and V191T.

Comparative analysis of the HRV-CU072 strain with most closely related strains

Table 14: Evolutionary relationship along ORF of HRV-CU072 compared with N4 strain.

Viral protein	Structural proteins				Nonstructural proteins						
	VP4	VP2	VP3	VP1	2A	2B	2C	3A	3B	3C	3D
Variation (nt)	55	209	176	213	81	63	316	59	20	139	397
Nucleotide variation (%)	27	26	25	26	19	21	32	26	30	25	29
Amino acid variation (%)	14	31	25	23	16	19	26	12	23	16	24
NS variation (aa)	9	80	56	63	23	19	34	9	5	29	70
NS variation (%NS)	19	58	42	44	41	43	19	20	36	25	30
NC-NS variation (%NC)	33	40	38	29	48	21	44	33	40	14	30

NS = nonsynonymous, NC = nonconservative amino acid

To elucidate the genetic relationship between the CU072 and other HRV-C, an estimated amount of synonymous and nonsynonymous variation at the protein level was investigated. In this analysis, nonsynonymous changes were defined as 2 types of variation: nonconservative and conservative nonsynonymous variation and were based on the presence or absence of changes in amino acid polarity. Sequence comparison of each individual protein precursor between CU072 and its closest relative strain N4 (GQ223227) indicated that the VP4 and 3A showed the highest overall sequence identity score (87%) whereas the VP2 represented the least conserved protein. The VP2 was found to have the largest numbers of both amino acid sequence variation (31%) and NS variation (58%) while the 3A region exhibited the lowest amino acid sequence variation (12%). Even though the

2A had less NS variation than the VP2 (41%), this protein displayed the highest percent NC-NS variation (48%). While the lowest NS score was found in the 2C (19%), this region had undergone profound NC-NS evolutionary change (44%) compared to other regions. Overall, the structural proteins of the CU072, especially in the VP1-VP3, showed a high average of nonsynonymous variability compared to the N4.

Phylogenetic relationship and recombination detection in HRVs

To observe changes in phylogenetic relationships, the PCM plot of nucleotide sequence alignment was performed using TreeOrderScan. The PCM results of each HRV species are summarized in Fig 28. HRV-A showed the lowest degree of phylogenetic incompatibility throughout the coding region, which correlated to a high level of sequence identity. The frequency of recombination in HRV-B and HRV-C was shown to be higher than HRV-A. HRV-C's phylogenetic relationship among species members had altered in the 2A and at the 3' terminal of 3D gene while the remaining regions remained conserved.

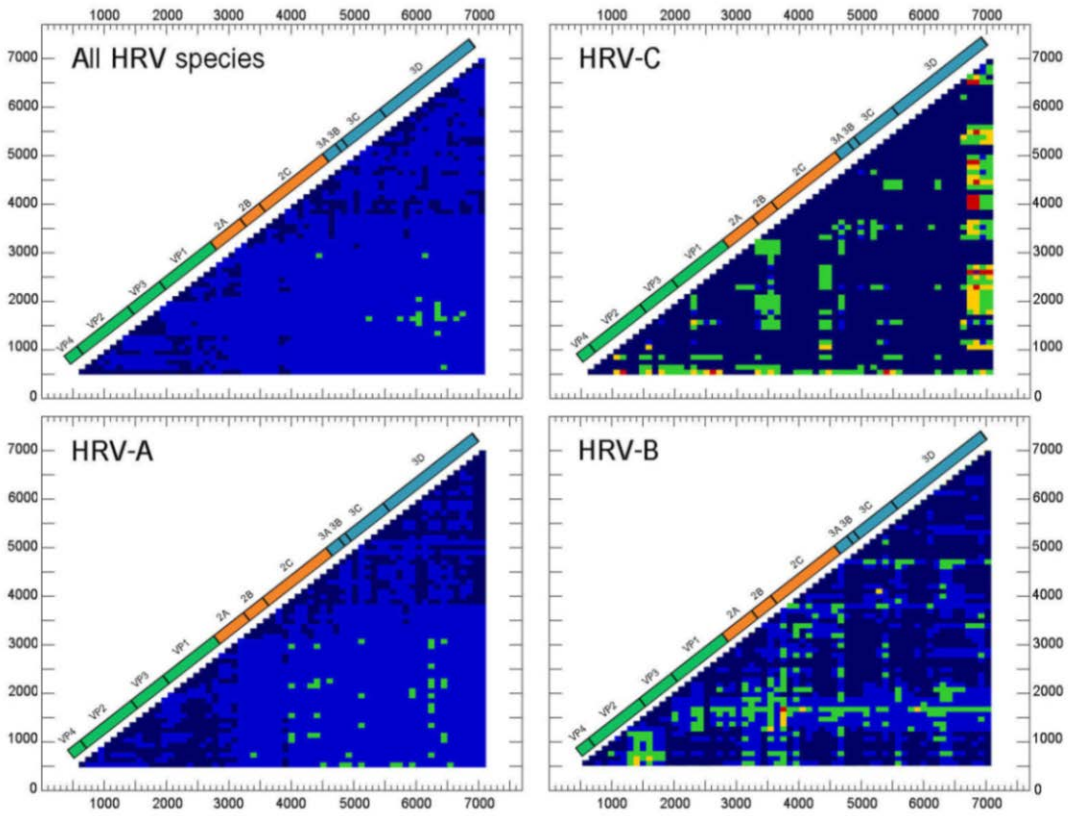


Figure 28: Phylogenetic compatibility matrices of HRV-A, B and C. Multiple sequence alignments of all known HRV prototypes including 6 identified sequences derived from this study were individually performed using TreeOrderScan. The numbers of phylogeny violation are color coded corresponding to an incompatibility frequency score of pairwise fragment comparison.

In order to determine HRV diversity and evolutionary characteristics, potential recombination events in the polyprotein gene were evaluated by comparison with all available HRV reference sequences. The results derived from a recombination detection program combined with similarity plot, bootscanning, and phylogenetic relationship suggested that CU211 was a new HRV strain derived from intra-species recombination of HRV-B. CU211 had arisen subsequent to multiple recombination processes within the HRV-B lineages. Most of CU211's coding sequence was similar to HRV35 (major parent: FJ445187) with 84% of pair-wise nucleotide sequence identity, while part of the VP2 and VP3 (positions 766-1590 nt) were genetically related to HRV69 (minor parent: FJ445151).

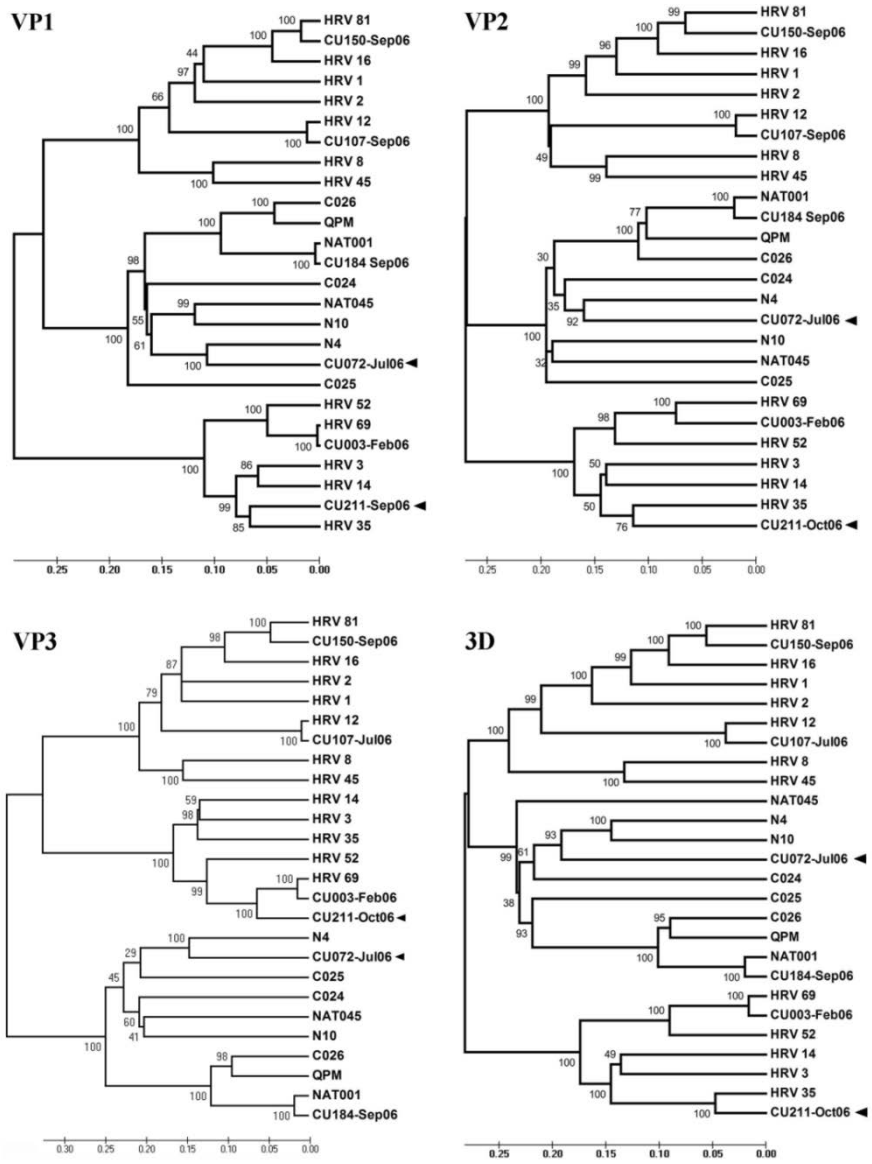


Fig 29: Phylogenetic analysis based on deduced amino acid sequences of VP1-VP3 and 3D of 6 identified strains compared with previously prototypes. Two new HRV strains are denoted by a black arrow.

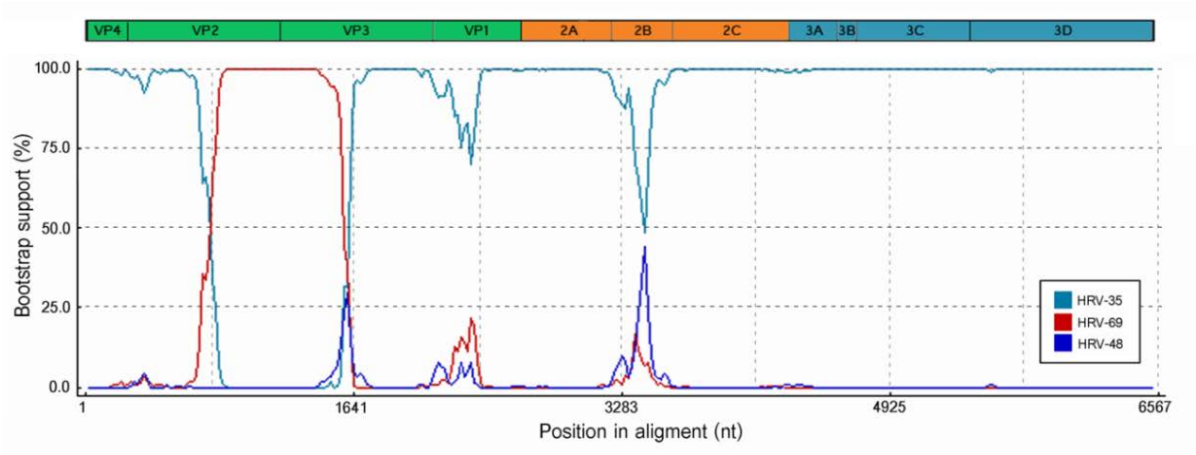


Figure 30: Bootscanning plot of recombination between the daughter strain CU211 and major (HRV35) or minor (HRV69) parental strains. Recombination breakpoint was predicted to occur at the ORF's nucleotide positions 766-1,590 covering partial VP2 and VP3 capsid encoding genes. Bootstrapping support value was computed using the RDP3 program with a window size of 200 bp, step size of 10 bp, and 1,000 replicates.

G+C content

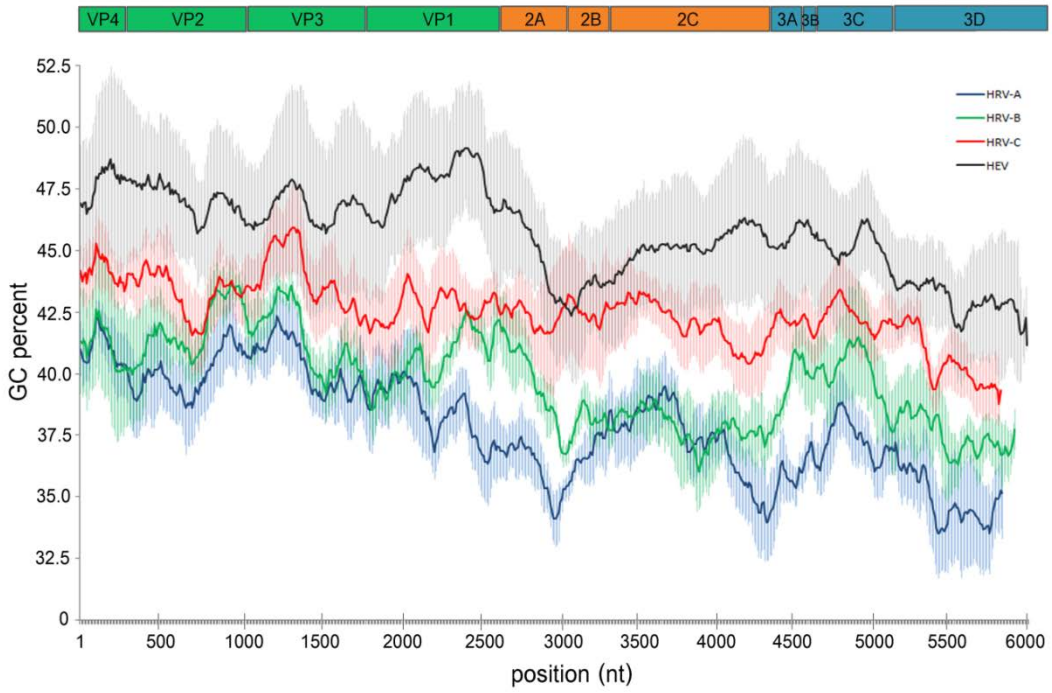


Figure 31: Average G+C composition percentage along the ORF of HRV and HEV. Each viral gene was depicted in relation to ORF arrangement. Average values were computed from multiple sequence alignments of representative serotypes or strains with each species by using 500 bp sliding window and 10 bp increment size. Standard deviation (SD) value of each species' representative data was represented by the shaded area.

Compared with the closest relative, all HRV species exhibited a lower percentage of average G+C composition than other HEV members (Fig 31). HRV-A and HRV-B showed a relatively low average G+C content (38% and 39%, respectively) whereas HRV-Cs displayed the highest average value at 43%. HRV-C's 2A cysteine-type protease encoding region showed a unique G+C content more similar to enterovirus composition than other HRV species. In comparison the other species, HEV-A and HEV-B, showed similar GC content (48%), PV displayed 46%, HEV-C 45%, and HEV-D exhibited the lowest G+C content at 42%, closely related to HRV-C.

Evolutionary rate, time of emergence, selective pressure, and origin of HRV and HEV68

1. Substitution rate and evolutionary timescale

1.1 HRV

To understand the evolutionary characteristics of HRV, this study estimated the divergence time of origin of each HRV species using a Bayesian MCMC method with the suit of model clock and population genetic method. The data set includes the recently described VP4/2 sequences sampled from this study and available nucleotide sequences of VP4/2 (300 sequence with 397nt) and partial 5'UTR (126 sequence with 400nt) of all HRV species with known collection or isolation date which were retrieved from the GenBank database (as of mid-October 2012). The homologous sequences of HEV were included in the data set and designed as out group. Using Bayesian MCMC method with a strict molecular clock for substitution rate calculation of VP4/2 gene, an overall evolutionary rate was estimated at $7.98x10^{-4}$ s/s/y with a 95% highest posterior density (HPD) of $5.59x10^{-4}$ to 1.04x10-3 s/s/y. Analyses incorporating the available sequences estimated for 5'UTR using exponential relaxed clock model showed that an estimated substitution rate of 5'UTR was slightly higher than the rate of VP4/2 ($1.16x10^{-3}$ s/s/y with $5.73x10^{-4}$ to $1.81x10^{-3}$ s/s/y 95% HPD). These analyses also indicated substantial incongruent phylogenetic relationships between viral genes. The results from evolutionary analyses of VP4/2 gene revealed that HRV-C and HRV-A share common ancestor together, while HRV-B was more closely related to HEV. The ancient HRV-C likely emerged apart from HRV-A around 121.93 with 95% HPD limit of 635.23 to 732.21 in VP4/2 gene. The tree topology recovered from the VP4/2 gene depicted the major bifurcation of circulating lineages of HRV-C indicated as HRV-C1 and HRV-C2 which were branched off from the most common

recent ancestor at 1258.87 (95% HPD of 1014.83 to 1458.03). By employing a Bayesian skyline coalescent prior, current circulating variants of HRV-C from several part of the world also formed 2 evolutionary lineages in 5'UTR (Fig 32) indicated as HRV-Ca and HRV-Cc with estimates of divergence time to be at 1361.27 with 95% HPD limit of 841.32 to 1731.65. Viruses belonging to HRV-Ca cluster were previously described as the HRV-C variants by which originated from the interspecies genetic sharing in the 5'UTR between HRV-C ancients and HRV-As. The analyzed results also showed that HRV-Ca was subdivided into 2 lineages and HRV-A represented as a sister taxa of HRV-Ca. This HRV-Ca and Cc lineages were interspersed within HRV-C1 and HRV-C2 clusters of VP4/2 genes. The divergence time when each HRV-Ca lineage branched off from their common was estimated at the early of 1500s and, thereafter, HRV-A was subsequently emerged at 1667.91 with 1442.34 to 1837.59 95% HPD.

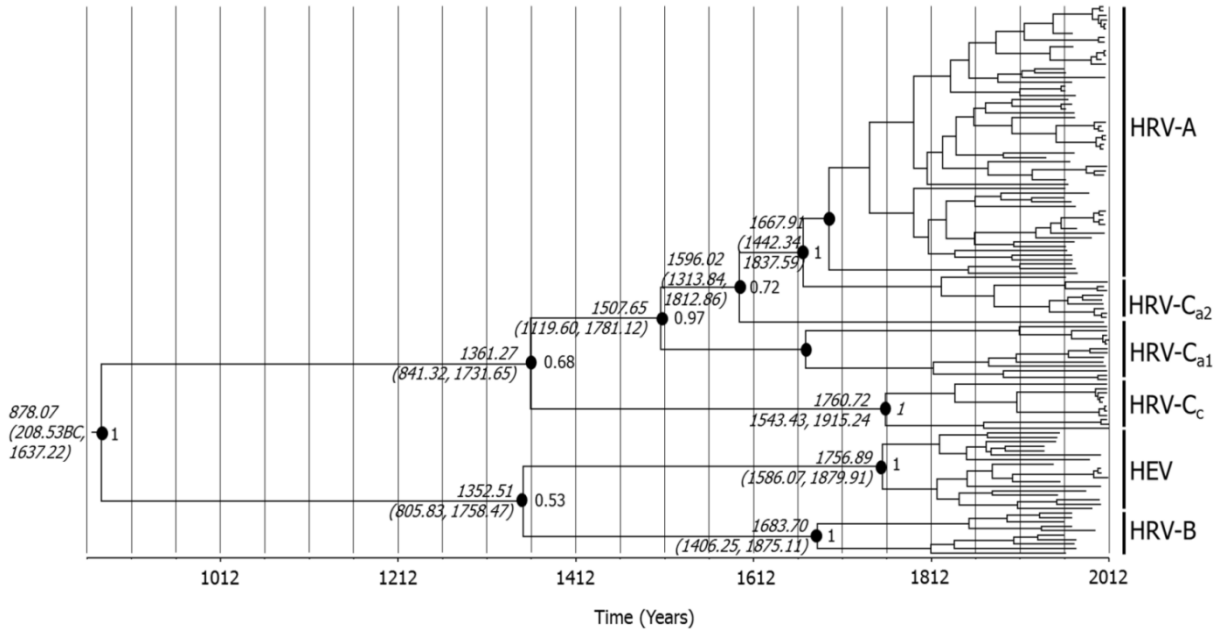


Figure 32: Dated phylogeny inferred with the HRV and HEV 5'UTR through Bayesian analysis implementing exponential relaxed molecular clock model. The names of sequences are not included in the tree for clarity. The virus species and major clades are indicated by vertical line at the right side and named. The divergence times from the most common recent ancestors are shown in each node with the mean posterior estimate and 95% Bayesian credible intervals of their ages. Time axis is scaled from the TMRCA to the present year (see larger figure in Appendix F).

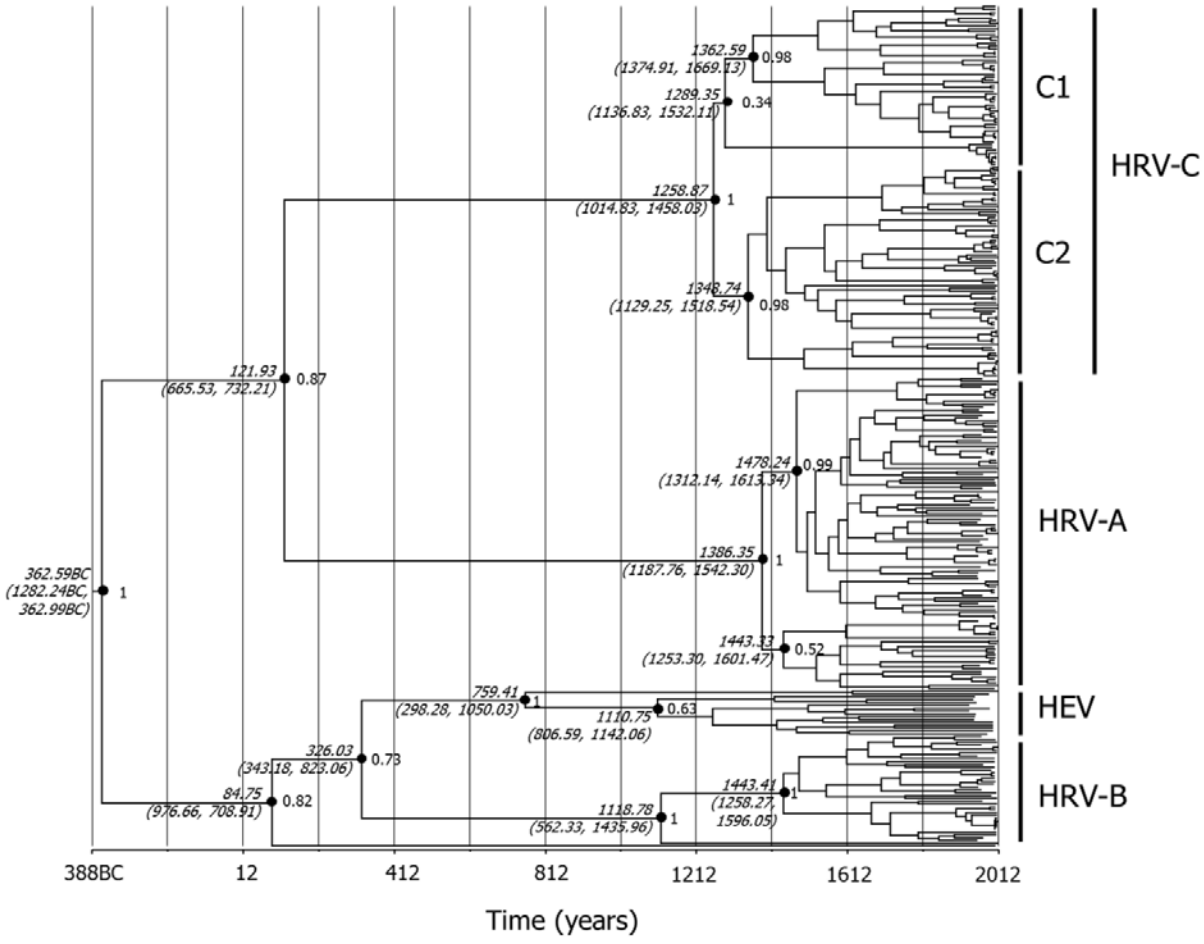


Figure 33: The maximum-likelihood phylogenetic tree of HRV inferred with VP4/VP2 sequences (300 sequences with 397nt in length) longitudinally sampled between 1959 and 2012. Each HRV species is classified into 3 species, denoted as HRV-A to C, respectively, with and the 2 clusters of HRV-C (C1 and C2). For clarity, the years of isolation and name of each sequence is not shown in the figure. The dates of the most common recent ancestors of each species and cluster were estimated using strict clock model and constant size coalescent Bayesian skyline prior as implemented in BEAST. Scale bar signify a time of divergence formatted to the present year.

1.2. HEV68

By using the Bayesian MCMC method, the overall evolutionary rate of the VP1 gene of the entire HEV68 sequence dataset (dataset 2) was estimated at 4.93×10^{-3} s/s/y with a 95% HPD limit of $4.01\text{-}5.85 \times 10^{-3}$ s/s/y. The evolutionary rate was lower (3.2×10^{-3} s/s/y with a 95% HPD of 2.27-4.25 x10-^{3} s/s/y) when the values were calculated with dataset 1 including a more homologous sampling timescale of all HEV species. According to the analysis of the evolutionary timescale of HEV68-VP1 sequences (Fig 34), the major

bifurcation of the currently circulating HEV68 strains from their first isolated prototype (Fermon) occurred 66 years ago (time 1945.31 with (1925.95-1960.46)95% HPD). Based on molecular signature analysis of the BC and DE surface loops of the viral determinant VP1 protein, 3 clusters of HEV68 were categorized with the pp value of 1 for clusters 1 and 2, and pp of 0.93 for cluster 3 (Fig 34). The date when each cluster branched off from TMRCA occurred in the 1970s (1975.78, (1946.13-1984.97)95% HPD). Furthermore, HEV68 seems to have diverged from their TMRCA with HEV species D 198 years ago (1813.42 with (1676.61-1920.46) 95% HPD).

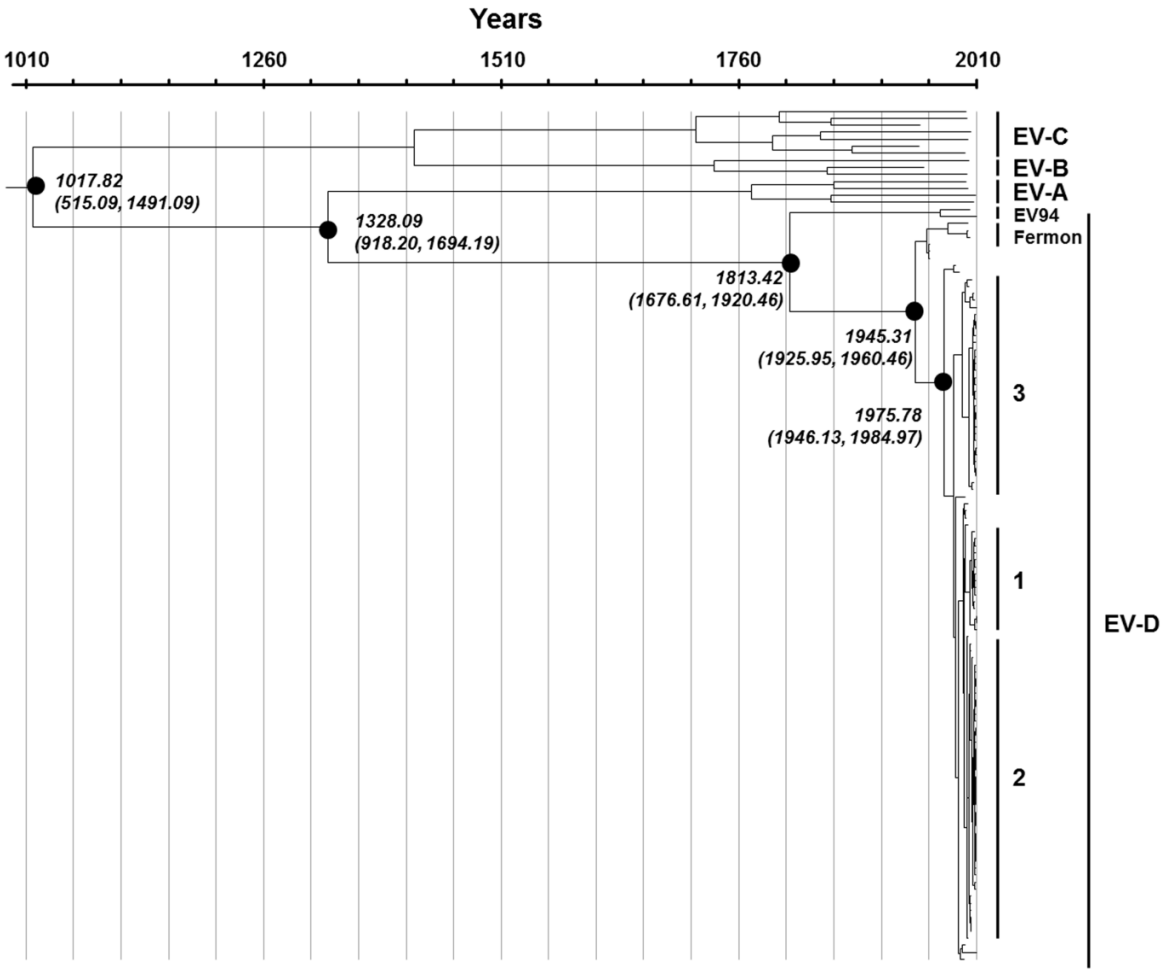


Figure 34: Bayesian time-scale phylogeny of HEV68 and other HEV based on the partial VP1 analysis (dataset 1 containing 122 sequences with 723 nt in length). Maximum clade credibility tree obtained with BEAST with a constant size coalescent prior showing lineage splitting events since the most recent common ancestor to the presently circulating HEV68 strains. For the TMRCA, the correspondent 95% Bayesian credible intervals are shown. Time axis is shown in years and ranges from the TMRCA to the present year.

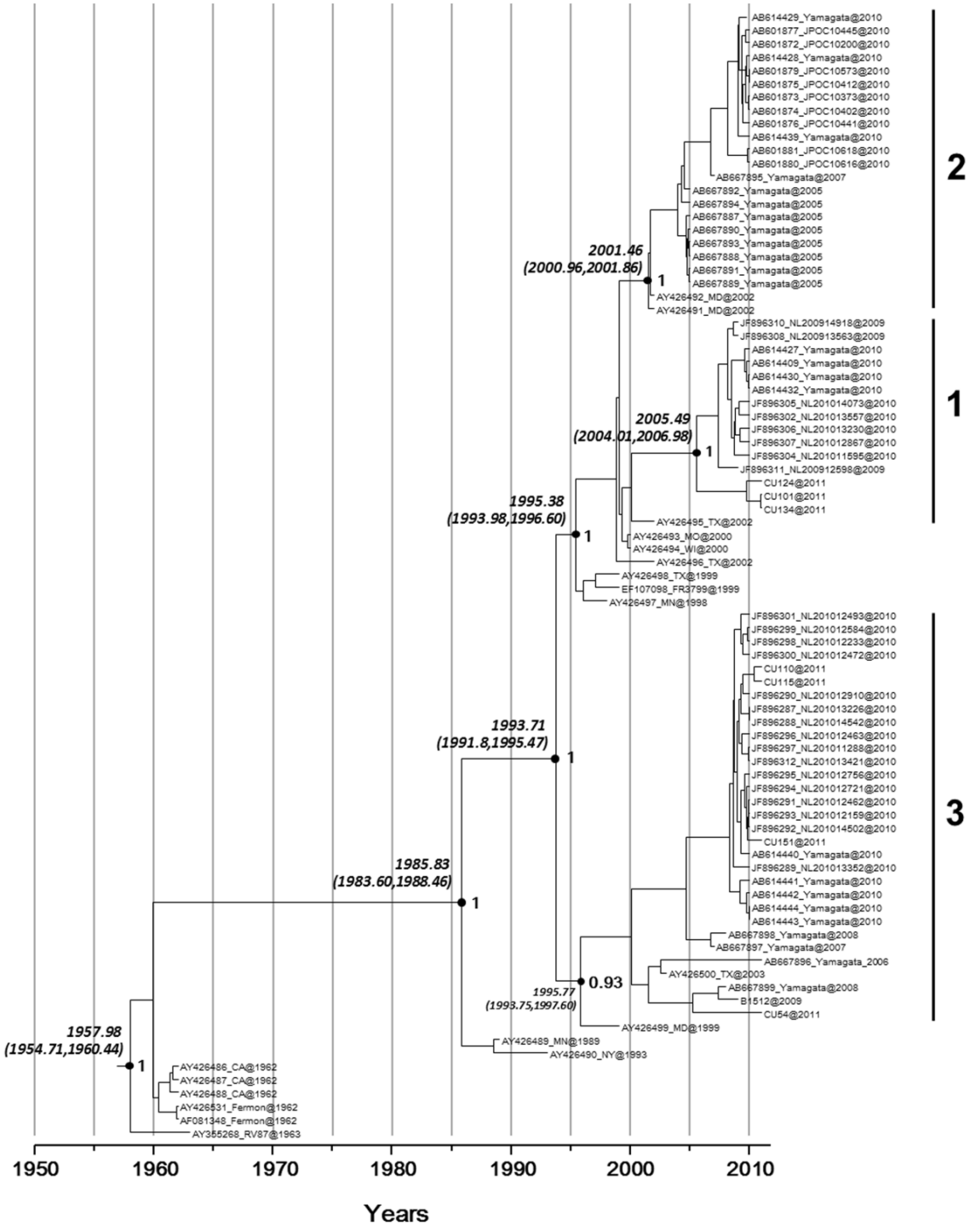


Figure 35: A maximum clade credibility tree from Bayesian timescale phylogenetic analysis of HEV68 (dataset 2; n = 106 sequences, length = 723 nt). The posterior probabilities and 95% HPD intervals of the key nodes are depicted above the respective nodes. Three major clusters are marked by vertical lines.

2. Detection of adaptive molecular evolution

2.1. Selective pressure analysis of HRV

This study utilized the available concatenated virus coding sequences, structural P1 and complete VP1 sequences of the prototypic strains of HRV-A (n=74) and B (n=25) and 14 coding sequences and 93 VP1 sequences for HRV-C to compute the selective pressure by using codon-based substitution method implemented in Datamonkey program. Selective pressure analysis on the coding sequence of all HRV species showed that majority of the virus genome part is driven by strong purifying selective pressure with the spike of codons under diversifying pressure in the external capsid protein VP2, VP3 and VP1 and 3D gene.

Table 15: Comparison of mean *dN/dS* at the individual sites of P1 and VP1 among HRV species.

Method	**SLAC**	
Species and region	**P1**	**VP1**
HRV-A	0.113 (0.112-0.114)	0.102 (0.099-0.105)
HRV-B	0.055 (0.053-0.058)	0.075 (0.070-0.081)
HRV-C	0.165 (0.161-0.169)	0.156 (0.151-0.162)
No of negative selection site of HRV-C[a]	327	216
Codon position of positive selection site of HRV-C[a]	594P1	276VP1
	FEL	
No of negative selection site of HRV-C[b]	431	236
Codon position of positive selection site of HRV-C[b]	341, 449, 594	-

Positive or negative selection site was determined by the value of ([a]) normalized dN/dS or ([b]) dN/dS > 1 and < 1, with $p<0.1$.

HRV-C accumulated the greatest genetic evolution in P1 capsid gene as it showed the highest dN/dS mean value followed by HRV-A and HRV-B (Table 15). The largest surface exposed capsid VP1 of HRV-A and HRV-B was a structural protein showed the greatest number of residue under diversifying pressure followed by VP2. In contrast, HRV-C showed extensive diversifying selection in VP1 followed by VP3 and VP2, respectively. Similar to the results observed in P1 analyses, the substitution ratio of HRV-C's VP1 was higher than the mean ratio of HRV-A and HRV-B. Additionally, some individual sites under positive selection were found in the study sampled VP1 data set using SLAC analysis,

including HRV-B at the position 92, 98 which were located at surface exposed-BC loop and position 282 and HRV-C at 276.

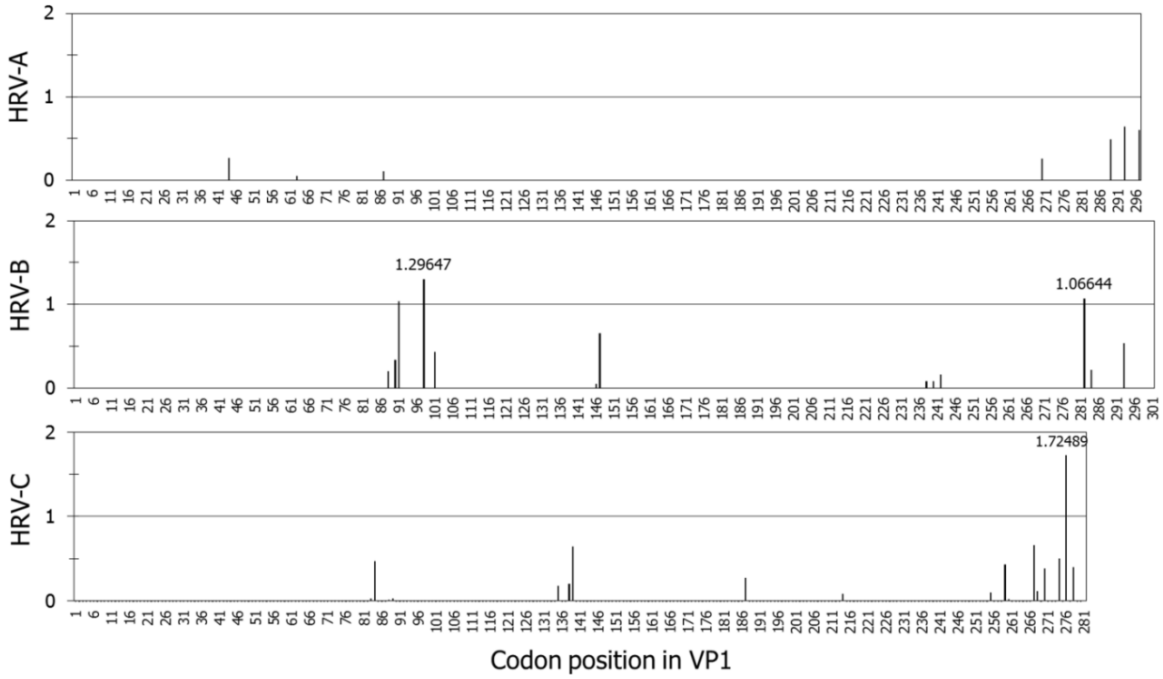


Figure 36: Ratio of number of non-synonymous to synonymous mutations (*dN/dS*) single amino acid step across the capsid VP1 inferred from the sequences of the prototypic strains of HRV-A (n=74) and HRV-B (n=25) and phylogenetic-assigned type of HRV-C combined with the recent circulating strains (n=93). Locations of codon under positive selection pressure are shown with the value of normalized *dN/dS i*ndicated above the graphs.

2.2. Selective pressure on HEV68 VP1 gene

The ω values per codon site across the partial VP1 region of HEV68 are shown in Fig 37. The overall ω value of the VP1 region was 0.091. The majority of amino acid residues in the VP1 region had $\omega < 1$ indicating that the amino acids in the antigenic determinant were under purifying selection. Results from FEL method revealed that codon position 23, 78, 86, 91, 93, 172, 212 and 237 displayed $\omega > 1$ without statistical significance. Codon positions 86 and 237 displayed 1.191 and 1.372 normalized dN-dS values, respectively. However, codon position 237 was the only residue that significantly evolved under positive selection ($p = 0.046$). The dominant amino-acid compositions at the alignment positions of BC and DE-surface loops of HEV68 are shown in Fig 38.

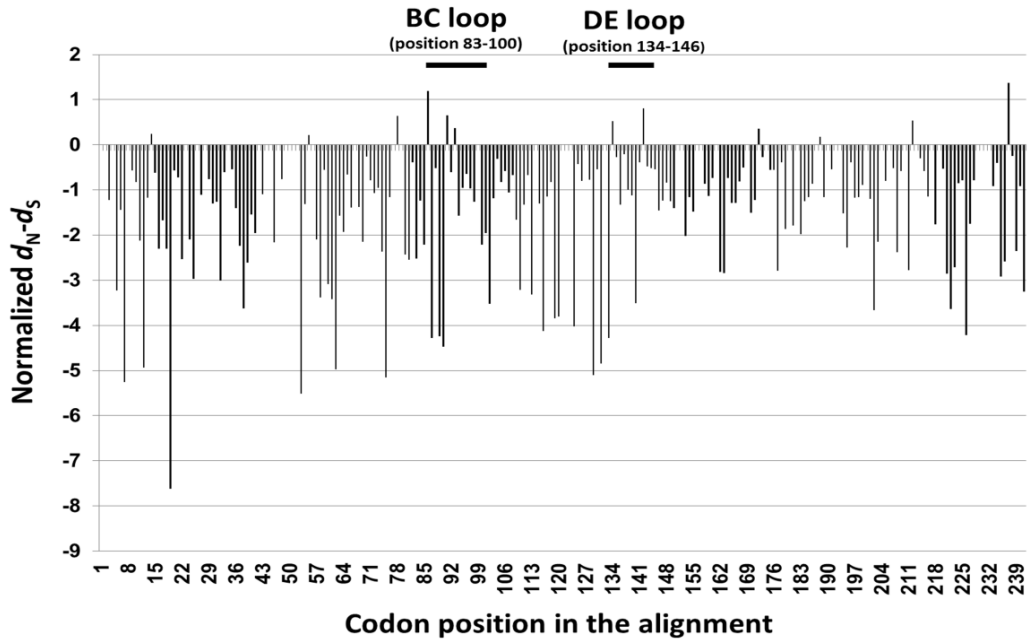


Figure 37: The ratio of nonsynonymous to synonymous substitutions (ω = dN/dS) per codon site across the VP1 region of HEV68. The residue likely to have evolved under positive selection was recognized when the ω > 1.0. Codon position assigned to this plot was based on the position of the VP1 protein in the HEV68 prototype, Fermon (AY426531).

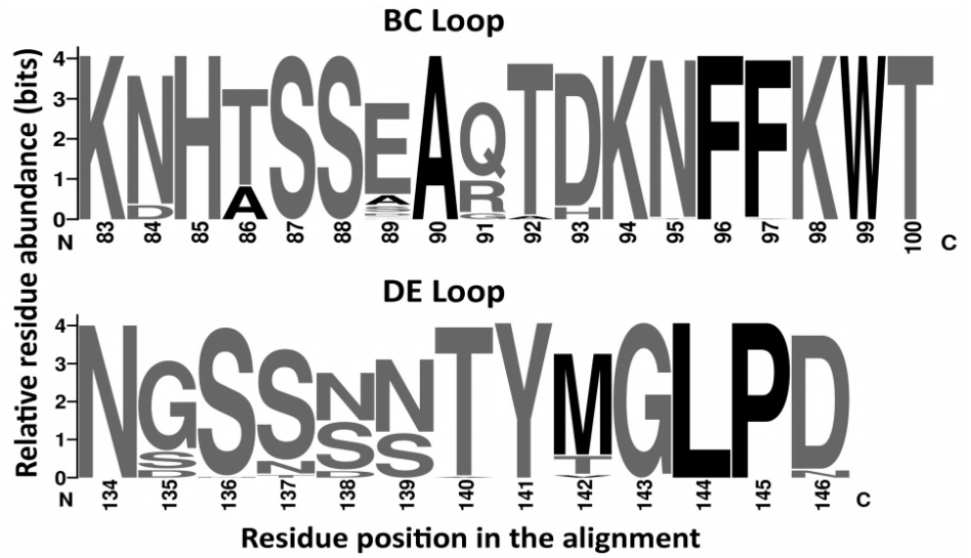


Figure 38: Relative residue abundance at the alignment positions of BC and DE surface-exposed loops. The graphical representation was generated using WebLogo. The height of symbol indicates the relative frequency of the corresponding amino acid at that position. Residue positions are given based on the nucleotide positions of the Fermon strain (AY426531).

CHAPTER IV

DISCUSSIONS AND CONCLUSIONS

All of the results in this study have been published in 2 different journals as following;
Publication 1: Complete coding sequence characterization and comparative analysis of the putative novel human rhinovirus (HRV) species C and B. Virology Journal 2011, 8:5.
Publication 2: Molecular Epidemiology and Evolution of Human Enterovirus serotype 68 in Thailand, 2006-2011. PloS ONE, May 2012, Volume 7, Issue 5, e35190.

Epidemiology, seasonality and genetic variation of HRV and HEV68

The successful of the epidemiological studies of HRV and HEV are depended on many factors such as the frequency of detection, appropriate population size, and reliable methods being used. Even though numerous PCR approaches have been widely conducted to study the virus epidemiology and clinical outcomes since the method was established in 1988, the effective reference method performed on the new identified types is still limited. A review of HRV studies between 2007 and 2012, which performed the virus detections on basis of capsid regions and 5'UTR-specific PCR amplifications, revealed different patterns of some HRV phylodynamic clustered within HRV-A clade (indicated as first HRV-A2), these viruses formed a clade of their own when capsid coding sequences were used [105-107, 140]. By using nucleotide sequences of structural gene regions of HRV as a predictable serotype, Susanna and colleagues suggested the discovery of new HRV species, labeled as HRV species C [141]. Findings from earlier observations have raised our concern about the reliability method suitable for HRV identification, and suggested that 5'UTR alone did not segregate the new identified HRV-C from their closely related HRV-A, and not quite suitable for identifying of the tentative predicted genetic types. Furthermore, positive results obtained from 5'UTR-specific PCR may be obstacles for accurate interpretations of the co-detection frequency between HRV and HEV. Therefore, in order to achieve an accurate description of the virus epidemiology, the utilization of PCR methods conducting more than one amplification such as 2 step-PCR (separate cDNA synthesis), nested- or semi-nested PCR of the same gene region is required [142]. Separate amplification of highly conserved 5'UTR in order to achieve pan-enterovirus screening combined with amplifications of the entire genome or partial capsid coding sequences of

VP4, VP4/VP2, or VP1 regions are suitable alternatives. Regarding to these concerns, sequence-based assay that target a fragment of two-third of the 5'UTR, the entire capsid VP4 and the 5'-end of VP2 gene (5'UTR/VP2) was reasonably developed using semi-nested PCR amplification and nucleotide sequencing strategies. Furthermore, by using the same primer set, the final PCR product of the specimens in which HEV68 implicated was 100nt-larger than the samples that contained HRV due to major deletion of HRV in 5'UTR. Thus, this PCR approach could be utilized as a broad and sensitive diagnosis tool for elucidating aspects of the molecular epidemiology of HRV and HEV.

This study retrospectively analyzed the epidemiology and seasonal model of HRV and HEV68 by using PCR amplification together with molecular genotyping and was one of the first detailed descriptions of comprehensive population-based surveillance of HRV and HEV68 among Southeast Asia countries and provided evidence for their significant roles in childhood ARTI. Of enrollment children aged less than 15 years old with ARTI complication, approximately 45% were infected with at least one respiratory viral agent. The analyzed results suggested that HRV being one of the most common viruses with annual prevalence of 13.8% among ARTI cases. Infections in both of URTI and LRTI patients predominantly targeted very young children particularly in infants and pre-school children with overall positive rates in children aged less than 5 years were 17% of ARTI, 22.6% of LRTI, and 11.4% of URTI. The most second targets of HRV infection were primary-school children and pre-school children suggesting the decrease of HRV activities with the increasing ARTI patients' ages. HRV was the major virus identified in young children with LRTI complications followed by RSV and Flu, whereas the main cause of URTI patients were identified as Flu infection. HRV circulating in Thailand are widely diverse, and the virus epidemics profile displays species, season, and year variations. The majority of HRV species identified in Thailand was HRV-C (48.08%) followed by the similar positive rate of HRV-A (28.2%) and HRV-B (23%). Interestingly, this study also showed an outbreak of HRV-B serotype 69 among URTI patients with the virus activities peaked during rainy season of the year 2010. Epidemiological surveillances from several countries worldwide suggested that circulation of HRV in human community did not usually caused by the predominant or outbreak of a single serotype. This was, for the first time, lead to the discovery of the persistence of specific HRV species and serotype. HRV-C was reported to be most frequently associated with LRTI [105, 109, 110, 140, 143-145]. On the other hand, the

majority of HRV species identified in URTI patients was HRV species B. Even though large-population based surveillance for both of LRTI and URTI patients were selected in this study, the final conclusion of the correlation between URTI severity and major HRV species could not be drawn and some analytical bias needed to be concerned due to an outbreak of HRV-B serotype 69 identified among URTI children. Moreover, the association between respiratory virus detection frequency and disease severity might be hampered by the global outbreaks of pandemic Flu-A virus 2009 [146, 147] which consequently caused high detection background of the virus and its relatedness. Other surveillance studies in tropical countries reported the emerging and re-emerging of several respiratory viruses [148-152]. Conducted over 27 years in Malaysia, a study screened common respiratory viruses in respiratory samples obtained from children under 5 years old with ARTI has been reported [153]. However, they used virus isolation and immunofluorescence staining as the routine detection methods instead of utilizing advance molecular amplification techniques. Thus, the considerably underestimated detection rate of respiratory virus they reported (25.4%) could be misleading and such information might not be suitable for further comparative analysis. In conclusion, respiratory virus illnesses caused by HRV infection are significant causes of morbidity in Thailand which predominantly found in young children. As suggested from the analysis of an association between ARTI and respiratory virus activity, relative humidity was the important climate factor reflecting virus transmission activity. Such information may be useful in an era of changing climate conditions worldwide and used for disease periodicity. This study also demonstrated that HRV-C has been circulating in human community for a long time with at least a decade. This analysis has gain insight into evolutionary dynamic and selective pressure across the virus gene. Ultimately, such knowledge may serve to elucidate the efficient therapeutics to reduce the clinical severity associated with this virus infection.

In recent years, with the use of highly sensitive molecular techniques, sporadically detected HEV68 virus has been recognized as a re-emerging respiratory pathogen in several countries worldwide since it has been detected with higher frequency in 2008 [11, 116]. HEV68 is becoming an increasingly important etiological agent as it was the only pathogen identified in respiratory specimens obtained from fatal cases reported in the retrospective studies of the Philippines and Japan [13, 111]. Interestingly, the presences of rare detected HEV68 were also demonstrated in this study. Twenty-five episodes of HEV68

infection associated with ARTI were investigated by phylodynamic relationship analysis, which was performed by evaluating nucleotide sequences of the region encompassing part of the 5'UTR/VP2 and VP1 gene. The analyzed results suggested that children above the age of 5 yrs. seem to be specifically targeted by EV68. Other studies also reported a similar correlation between patient's age distribution and frequency of HEV68 infection [11, 45, 111, 115]. Despite HEV68 infections generally affecting younger children, the association between virus infections and respiratory illnesses in adult patients has also been established [45, 116, 154]. In concurrence with the earlier reports, infection by this virus appears to be an important cause of viral pneumonia in children requiring hospitalization with common clinical presentations of fever, cough, dyspnea, and wheezing [13, 45, 116]. Among children with a history of asthma who experienced more severe asthma attacks, HEV68 was the sole etiological agent detected indicating its role as an aggravating factor to severe persistent respiratory disease. Furthermore, a strong association of asthma exacerbation and the virus infection has also been reported [12].

In Thailand, no epidemic status of HEV68 infection has so far been reported. Screening results showed that HEV68 probably emerged in Thailand in 2009. Since that time, HEV68 infected cases have more frequently been detected during 2010 and, thus far, displayed the highest prevalence in 2011. However, the virus activity in Thailand has ceased in 2012. Recently, two studies in Asia continent have reported the detection of HEV68 in patients with respiratory illnesses: (1) one research group identified 17 HEV68 infections in 816 severe pneumonia pediatric patients at the Philippines (2.08%) during 2008 to 2009 [13], and (2) the other group reported the study in Osaka, Japan, where 14 of 448 patients with ARTI and 1 with febrile convulsion had HEV68 as most likely cause of illness (3.12%) with detection period at 2010 [111]. Taken reports above together, these could suggest that reemergence of HEV68 could introduce to human community, at least in Asia countries, in 2008 [11-13, 45, 111, 116]. The finding in this study supported prior observations that the upsurge of HEV68 infection has apparently occurred on a global scale since 2010. Unlike other respiratory virus including HRV, detection and identification of HEV68 in respiratory samples are not incorporated into routine clinical screening due to its previously recognized as rare respiratory agent which could lead to the underestimation of the prevalence of HEV68 worldwide. In contrast to the low detection of HEV68 in clinical and environmental samples, seroprevalence studies have shown a high

percentage of neutralizing antibodies against HEV68 [112, 155], suggesting that HEV68 infection may common among human community and may be underrepresented in clinical samples because of mild or subclinical consequences. Distinct seasonal patterns of HEV68 infection in ARTI patients were found in Thailand during the study period. Infection rates were higher during the rainy seasons of 2009 and 2011 while no seasonal pattern was apparent in 2010. The variation in HEV68-seasonal profile examined in this study was similar to the profiles of respiratory viruses in tropical countries with a higher average temperature throughout the year and less changes in seasonal temperature [10, 125, 152, 156]. Other studies performed in temperate climates observed seasonality of HEV68 in the autumn seasons (Japan 2010 [111], France [157], Netherlands [116], and Italy 2008[158]). These observations suggested that the seasonal profile of HEV68 may vary by season, geographic location, and year.

Clinical manifestation of ARTI patients with HRV or HEV68 infection

Associations between virus serotype and species and clinical manifestations have not been conclusively established. Different HRV and HEV serotypes can cause the same symptoms, and the same serotype is often reported to cause multiple syndromes. Some epidemiology reports showed that URTI patients were more frequently infected with HRV-A and HRV-B. On the other hand, LRTI patients were reported to be the most frequent group associated with HRV infections notably viral pneumonia, bronchiolitis and exacerbation of asthma, with statistically significant supported. Furthermore, death and severe respiratory tract infection cases were either solely caused by HRV-A or HRV-C infection and/or by co-infection with other pathogens with the exception of HRV-B. ARTI patients infected HRV were found to fully recover from the illnesses with no evidence for long-term individuals' continuity or repeated infections with the same serotype. Employing phylogenetic comparison between 5'UTR/VP2, HRV-C shows the most genetic variability compared with HRV-A and HRV-B. Hence, descriptions of these high frequencies HRV-C detections among ARTIs can be delineated by their genetic heterogeneity and the possible high transmissivity.

While the current frequencies of HRV detection among pediatric patients are on the increase, worldwide HRV-B detection frequency is moderate to low when compared to other species. In concurrence with the earlier reports, low detection rate of HRV-B was

also found in his study. Interestingly, in an epidemiology study of 2010, HRV-B became a predominant species circulating in this year coinciding with the influenza season. According to high nucleotide sequence identities, most of the HRV-B strains circulating in Thailand belonged to serotype 69. Although the HRV circulating do not usually caused by the predominant or outbreaks of a single serotypes, this study, for the first time, found a recurrent outbreak of specific HRV serotype in Thailand during the rainy season 2010. Intra-species comparison also showed profound homology among HRV-A suggesting the stable evolutionary status of HRV-A and B whereas HRV-C displays a higher nucleotide divergence. In this current study found that the detection frequency of HRV in LRTI subject is higher than the rate of URTI subjects reflecting the important role of this virus in the development of more severe clinical complications. Nonetheless, there is some limitation of the assay need to be considered. Even though this study utilized a highly sensitive semi-nested PCR method, the virus yield and the sensitivity of virus assay in different types of samples might be an obstacle to conclusion as suggested by an earlier investigation.

Of the ARTI patient who had multiple hospitalizations, HRV repeated infections were found in 6 pediatric patients; 3 cases were URTI and their other 3 were found to be suffered from LRTI. For example, 2 patients were presented with severe LRTI and recurrent wheezing associated with repeated infections of distinct HRV strains and/ or species. Virology analyses of these 2 cases revealed that one of them, an infant girl who had 4 episodes of LRTI, was invariably by different HRV-C strains. HRV infection was identified for the first time in 14 months old by HRV-C co-infected with RSV, and it was found again at 21 months old with Flu-A and distinct HRV-C co-detection for the second time. Another incidence of recurrent wheezing patient was found in an infant boy who experienced re-infections by distinct HRV species; the first time at 7 months old, second time at 9 months old with distinct HRV-As, and the third time at 9 months old with 8 weeks apart from the second time with HRV-C. These observed findings warranted further investigations for several reasons. In general, HRV infection patients are capable to fully recover from respiratory illnesses within 7-10 days after being infect. Viral shedding can occur for a few days before the cold symptom onsets which normally peak during days 2-7 of the illness. In some cases, the virus titer may last as long as 3-4 weeks even though the symptoms have already ceased several days before. During the infectious period, increasing titers of specific immunoglobulin (Ig) G and IgA which are responsible for the virus controlling can

be measured with the coincidence augmentations of neutralizing antibody. The experimentally administrated HRV in healthy volunteers revealed that local specific Ab titers remained evaluated for at least one 1 year. Results shown here supported the different findings observed in this present study; (1) the duration of repeat infections were relatively occurred in a short period of time (2) these two patients experienced repeat infections by the distinct HRV strain of the same species. These phenomena could be described as HRV has high genetic heterogeneity and antigenically distinct and, thus, the possession of specific antibody raised against one species infection might not sufficient to provide heteroserotype cross-protection and disease reduction during the episode of HRV re-infection.

Global outbreaks of Flu in 2009 and 2010 resulted in a high overall incidence of respiratory illnesses. In Bangkok, Thailand, the number of ARTI cases was increased sharply after June 2009 as experienced in the first epidemic wave. However, high proportion of respiratory samples testing negative for both of Flu-A and Flu-B was found during the viral screening. A wide range of etiological agents causing ILI symptoms have been identified. Specific viruses exhibit particular epidemiological characteristics and distinct seasonality which can even show differences between the study areas. A number of epidemiological studies have suggested that HRV is one of the viruses hypothesized to be capable of interrupting the spread of Flu during a pandemic followed by RSV, Paraflu, and HMPV with sporadic findings of other HEV and AdeV. Results demonstrated here also indicated the possible delay of the spread of Flu by the increase of the activity of HRV during some specific weeks of rainy and winter seasons. These results were in accordance with previous observations for the potential of an epidemiological interference during the Flu pandemic waves in several European countries. For instance, in France, HRV infection appeared to reduce the probability of pH1N1/2009 infection, and affected its spread at the beginning of autumn. Furthermore, one study from Sweden team suggested that the warm and humid climate of late summer and autumn resulted in increased HRV infections which might interfere with the aerosol transmission of Flu. Similar to other European countries, Norway also experienced delayed Flu pandemics and found a relatively high proportion of HRV co-circulating with Flu. However, similar percentage of pH1N1/2009 and other community-acquired respiratory viruses in ARTs was demonstrated suggesting co-circulation of these viruses were represented a phenomenon of viral strain displacement

or viral interference. In order to gain insight into the role of virus interfering effects, surveillance of respiratory infections on larger cohort should be conducted throughout the year to create reliable baselines for ARTIs, which will be useful when a pandemic virus occurs and that does not follow the usual pattern of spread.

Despite data on association between meteorological parameters and respiratory virus infections have been reported in several countries, the data of respiratory virus activity, particularly HRV activity, in Southeast Asian is relatively understudied. Although variations in weather temperature and season in Thailand is minor, respiratory virus circulations appear to depend on relative humidity, rainfall and number of rain day transmit without specific transmission pattern. This is in accordance with the previous observations in tropical climate countries. Surveillance study in Brazil has suggested that relative humidity is an important factor associated with respiratory virus infections in community-acquired pneumonia patients. In this recent study, while, direct correlation between the activities of RSV in LRTI subjects with relative humidity were found as with in Indonesia, inverse correlation in the virus transmission was reported in Singapore and Malaysia studies. Among these analyzed climate factors, the best-fit parameter for the data was relative humidity determined by multiple regression analysis with stepwise method. Regarding the relative humidity, this study also established a simple regression equation models and validated these seasonal model for HRV activity in URTI and overall total number of LRTI predictions and validity of these seasonal models was examined by using the data gathered during the study period. The seasonal models showed correlation between the predicted peak level and reported number of the cases, although the peak level did not exactly match in some months. Limitations in accuracy of the prediction model could be explained by the typically minor variation in meteorological factors in tropical regions. Furthermore, the sharply increase of the number of respiratory samples during the course of global Flu pandemics has caused weak correlation in this analysis. Other explanations for different patterns of respiratory virus epidemiology and seasonality may include the quality of indoor air. Due to the heat and humidity in Thailand, Thai residences especially people living in Bangkok are more frequently exposed to air-conditioned environment than in natural ventilating environment resulting in more easily infected by respiratory viruses. Although the air quality alone is not the cause of the infection, this factor may increase respiratory symptoms and prolong the recovery of

wellness. The low indoor and outdoor ventilation in air-conditioned places may increase indoor-related pollutants and enhance the cough symptom and LRTIs especially wheezing and asthma. Further large-scale prospective cohort studies from multiple centers, epidemic areas and study years will be required on order to improve the seasonal model accuracy and to better understand the correlation between the virus infection and climate factors in tropical climate country.

Upsurge of patients with serious respiratory illnesses associated with HEV68 infections have raised the attention into this rare detected virus. Results obtained in this study suggested that children above the age of 5 years seemed to be the specifically target of HEV68 infections with distinct seasonal patterns of HEV68 infection in ARTI patients. The variation in HEV68-seasonal profile examined here was similar to the profiles of respiratory viruses in tropical countries with a higher average temperature throughout the year and less changes in seasonal temperature. Infection by HEV68 virus appears to be an important cause of viral pneumonia in children requiring hospitalization with common clinical presentations of fever, cough, dyspnea, and wheezing. Among children with a history of asthma who experienced more severe asthma attacks, HEV68 was the sole etiological agent detected indicating its role as an aggravating factor to severe persistent respiratory disease. Furthermore, a strong association of asthma exacerbation and the virus infection has also been reported. The association between virus genetic variation and its epidemiological characteristics has been documented. Phylogenetic relationship and sequence analysis of capsid VP1 displayed three distinct phylogenetic clusters with unique molecular signature in surfaced exposed BC and DE loop located on the viral determinant VP1 protein. Furthermore, prototypic strains of HEV68 were found to have phylogenetic clusters separated from the recent identified strains [45, 116]. These results strengthened the hypothesis of a change in the epidemiological and clinical spectrum of HEV68 infections by changing the circulating lineage which might contribute to a rise in more serious respiratory illnesses. Despite having performed this study on stored specimens collected since 2006 and utilizing sensitive PCR approaches, some limitations need to be considered. First, it was possible to conclude that ARTI associated with HEV68 infection was first introduced to Thailand in 2009 since HEV68-infection was not found between 2006 and 2008. Nonetheless, the limited number of specimens towards the end of 2008 and during the first 5 months of 2009 might be an obstacle to conclusion of this

longitudinal study. Second, although this study utilized specimens which collected from several hospitals located in many different regions, at least 80% of children were Bangkok residents. Therefore, the enrolled children might not be representative of the entire country population. Increased surveillance together with improved laboratory diagnostic techniques will help reveal epidemiology, pathogenicity and clinical association, and the worldwide circulation of this virus.

Genetic characterization of the putative new HRV-C

In this study determined the complete coding sequences and summarized the molecular characteristics of a putative newly identified HRV-C strain. Furthermore, this study report a new HRV-B member derived from intra-species recombination. In the absence of serological neutralization data of HRV-C, the HRV-C variants can be classified into 33 genetically-defined types based on divergence thresholds calculated from the distribution of pair-wise sequence distance. Results obtained from the HRV-CU072 strain showed it exhibited a low sequence similarity score (36% sequence divergence) and a distinct evolutionary phylogenetic relationship to the HRV-C criteria proposed by Simmonds et al [159]. Several typical enterovirus and rhinovirus sequence characteristics are still conserved in HRV-CU072, such as potential utilization of the ICAM-1 protein as its specific receptor and possible resistance to synthetic pleconaril. However, this strain displayed some unique properties as for example, it uses a VP3/VP1 (N/D) cleavage site predicted by distinct alignment. Several studies on HRV, HEV and other picornavirus genera have examined variation across their genomes [85, 87, 160]. In HRV species, the structural proteins VP1, VP2 and VP3 and the nonstructural 3C and 3D proteins have been identified as diversifying selective regions that are thought to influence the evolution of HRVs. Although the capsid region is prone to high NS variability, the HRV-CU072 strain has conserved the essential motifs such as receptor interacting site and drug binding pocket along with other HRV-C members.

This study compared nonsynonymous and synonymous substitution at the protein level of the HRV-CU072 strain with its phylogenetically closest relative (N4 strain) to elucidate the evolution of this newly identified strain. Analysis results suggested that the degree of sequence variation between them might not necessarily be ascribed to their genome size. Although the HRV-CU072 capsid region displayed high NS variation, the

essential motifs such as receptor interacting site and drug binding pocket were conserved as in other HRV-C members. The VP4 capsid protein showed the highest sequence identity score compared with others. Due to its function as an internal surface protein VP4 is not involved in rhinovirus antigenicity. This might explain why the VP4 protein is highly conserved and shares familiar characteristics among the HRVs and HEVs. The analysis results revealed that the HRV-CU072 and N4 strains are descendants of a recent common ancestor via the purifying selection mechanism on the structural genes. In addition, this could suggest that the HRV-CU072 strain is not an N4 variant and might be a putative new HRV-C strain.

Ubiquitous recombination in HEV and other picornavirus genera such as *Aphthovirus* and *Teschovirus* has been well established as an evolutionary driving force [89, 161-164]. Despite its overall genetic similarity to HRV, HEV recombination frequently takes place in either the nonstructural (mostly P2) region, or between the 5´UTR and adjacent capsid coding region. This results in a limited set of capsid genes responsible for HEV serotypes [161, 164-166]. Many previous comparative studies have concluded that recombination in HRVs can occur throughout their genomes. The sites most favored for recombination have been frequently reported to occur in the noncoding and nonstructural regions [28, 87, 89, 105, 167]. In concurrence with the earlier reports, the results form PCM analysis described in this study also showed the overall recombination breakpoint of HRV species can randomly occur throughout the coding sequence. The PCM results of each HRV species illustrated that the different HRV species showed different degrees of phylogenic variation, representing a unique species-specific property. Interestingly, HRV species A exhibited a high degree of phylogenetic compatibility with each other within the capsid genes, 2C and nonstructural P3 regions. This indicates that the intra-species recombination processes of HRV-A were probably limited to these parts of the genome. In addition, all HRV-A members shared genomic characteristics conserved within the species and inter-species recombination was probably limited.

Huang *et al.*, 2009 [106] and McIntype *et al.*, 2010 [107] have reported that HRV-C showed evidence for inter-species recombination with HRV-A exhibiting 2 precise recombination hotspots in the 5´UTR and 2A gene. For the new species, HRV-C, PCM analysis results showed that sequence variations within HRV-C have been prone to accumulate in some genomic regions, particularly in the nonstructural 2A gene, as has

been recently reported [107, 167] and probably in the 3D coding gene which might influence the dynamic process resulting in intra-species C diversity. From findings in this study it could be concluded that the 3D gene encoding the RNA-dependent RNA polymerase is the site favored by HRV-C for recombination. Only a few reports have indicated recombination in circulating strains. Recombination has recently been demonstrated between circulating heterogeneous HRV-A and some HRV-C strains. Palmenberg *et al*. reported an intra-species recombination in HRV-A which resulted in the origin of a novel clade D virus. Tapparel *et al*. [58] observed phylogenetic incompatibility in the 5´UTR, VP1 and 3CD regions of 2 HRV-A strains. Huang *et al.* [106] have also described HRV-A intra-species recombination events among 3 field strains with phylogenetic incongruency in the 5´UTR and VP4/VP2 regions and 2 HRV-C field strains have arisen from inter-species recombination with HRV-A. This study suggests an infrequent recombination event among HRV-B lineages (HRV-CU211) identified from an acute lower RTI patient diagnosed with viral pneumonia with recombination breakpoints at the boundary of the capsid encoding VP2 and VP3 genes. Although recombination events occurring in some parts of the different RNA genomes have not been recognized as a major mechanism for HRV evolution or as crucial for the large diversity of HRV circulating in humans, this process is still utilized for diversifying genome sequences. Furthermore, the detection of the recombinant strain in LRTI patients may raise concern about the correlation between recombination and change in disease severity.

Studies on base composition in viral genomes can provide molecular information and thus contribute to understanding the efficient regulation of viral gene expression, codon usage bias, viral genome stability, and replication capability. Such information would also be relevant to elucidate their molecular evolution. Mutation pressure and composition constraint, particularly in G+C content, of the viral RNA genome are often considered important evolutionary genomic factors accounting for variations in codon usage among genes in different organisms [58, 168, 169]. In parallel with the molecular characteristics of HRV and HEV species, the average G+C content of their genomes has previously been described as a genomic factor to explain differences in RNA stability, optimal growth temperature, tissue tropism and also disease pattern. In HEV, a high G+C content of the viral genome is thought to be an essential factor for HEV's adaptive capability to replicate in various parts of the human body including respiratory tract,

gastrointestinal tract, and CNS. In contrast, the most closely related HRV species exhibited a lower G+C content than other enterovirus members which might reflect their adaptation to the lower temperature environment and sensitivity to the gastrointestinal tract's acidic pH. In this study, we found similar G+C content values of HRV-C and HEV-D coding sequences, contrary to the relatively low values in HRV-A and HRV-B. This may reflect HRV-C's capability to adapt to the higher temperature environment of the lower part of the human respiratory tract and thus differentiate it on some phenotypic level from other HRV species. This finding might also support several epidemiological studies on HRV in that HRV-C was more predominantly found in acute lower RTI cases than HRV-A and HRV-B and may significantly contribute to severe respiratory tract disease development, especially the exacerbation of asthma and wheezing. However, sequence analyses of other picornaviruses such as human hepatitis A viruses, hepatotropic members of the genus Hepatovirus, which replicate primarily in the gastrointestinal tract and spread to the liver causing liver failure and jaundice have shown a much lower G+C content [170]. To further understand this finding and investigate the mechanisms of virus-induced asthma exacerbations, HRV-C's mode of infection should be further investigated.

Little is known about the association between adaptive mechanisms and HRV evolution. The results have provided information on the role of selection pressure and recombination mechanisms influencing the evolution of HRV. Further studies should be performed to better understand the clinical impact of each species on respiratory disease, epidemiology, their genomic characteristics, and the mechanisms controlling variation and evolution of this virus.

Evolutionary history, origin and selective pressure analysis of HRV and HEV68

This study showed analysis of HRV and HEV68 provide some insights on the evolutionary history, the origin and evolutionary force influencing the virus diversity. To address this concern, this study estimated the divergence time of origin of each HRV species and HEV68 using a Bayesian Markov chain Monte Carlo method with the suitable model clock, population genetic method and well sample representative sequence data set of VP4/VP2, VP1 and/ or partial 5'UTR sequences of all HRV species and HEV68 with known collection or isolation date. Molecular clock for substitution rate calculation of the VP1 determinant indicate that the rate of evolutionary change estimated for HRV is faster

than the mean evolutionary rate of that observed in HEV68 ($4.93x10^{-3}$ s/s/y). This value was in accordance with the evolutionary rates of other newly emerged picornaviruses such as HEV71 genotype B ($4.2x10^{-3}$ s/s/y), HEV71 genotype C ($3.4x10^{-3}$ s/s/y), and human parechovirus ($2.8x10^{-3}$ s/s/y) and hepatitis A virus (9.8x10-4 s/s/y) [171-174] whereas it is lower than the rate of PV ($3x10^{-2}$ s/s/y) [82, 175]. Results obtained in the present study also suggested HEV68 could be divided into 3 clusters based on their phylogenetic relationship and the unique molecular signatures within the BC and DE surface loops which was supported by high values of pp. This suggestion also resembled with the report of Meijer and team [176].

Consistently, the high evolutionary rate among these viruses relied on several factors including replicase fidelity, highly error prone viral RNA-dependent RNA polymerases resulting in a misincorporation frequency of 1 per 10^3 to 10^4 nucleotides, rate of transmission, and any synonymous mutations of viral proteins [177, 178]. These factors increased the number of mutations incorporated in viral genomes overtime and prepare the ground for rapid genetic diversification. Moreover, intra- and inter-serotype recombination was also believed to be a large-impact evolutionary mechanism influencing the genetic and antigenic diversity of HEV, PV, and other picornaviruses [57, 76, 77, 86, 89, 100, 106, 107, 161, 163-165, 167, 179-181]. Furthermore, this study indicated substantial incongruent of genetic evolution between viral genes. Comparative analyses of representative sequences of HRV inferred that 5'UTR accumulated the highest substitution rate ($1.16x10^{-3}$ s/s/y) and was slightly higher than the rate of VP1 and VP4/VP2 gene ($7.98x10^{-4}$). Similar substitution rate of crude HRV's VP4/VP2 gene was obtained by the study of Briese and colleagues ($6.6x10^{-4}$ s/s/y) even the different molecular clock model was used [182].

HRV-C and HRV-A shared common ancestor together and represented as sister taxa of HRV-B, which was more closely related to HEV. Previous conservation suggested that some of HRV-C variants showed phylogenetic compatibility and high sequence similarity in 5'UTR when compared to HRV-A while the remaining genome parts showed phylogenetically distinct features from other species [105, 106]. This implied that those HRV-C variants interspecies genetically recombined with HRV-A variants, with recombination breakpoints located in the stem-loop 5 and polypyrimidine tract of, resulting in classification of 2 separate clusters of HRV-C, Ca and Cc [107]. This theoretic

interspecies recombination had been proved by interchangeable of the 5'UTR between the chimeric HRV-A and HRV-C viruses. The distinct evolutionary histories in 5'UTR and VP4/VP2 were found in this recent study. By employing a Bayesian skyline coalescent prior, current circulating variants of HRV-C from several part of the world formed 2 evolutionary lineages in 5'UTR, indicated as HRV-Ca and HRV-Cc, with estimated of divergence time to be at 1361.27. HRV-Ca was subdivided into 2 lineages with the divergence time estimated the early of 1500s and HRV-A represented as a sister taxa of HRV-Ca (emerged at 1667.91). Taken evidence together, recent findings shown here might imply that this new HRV species is not a recently emerged human pathogen. Otherwise, it has been evolving from a probable descendent of the closely related HRV-A and circulating for more than 750 years ago before the emergence of HRV-C circulating recombinant variants. Evidence supported that HRV-C has been circulating cryptically in human community for at least a decade with remarked high prevalent and genetic diversity have been reported elsewhere [22, 183-185]. Description of the underestimate of detection rate of HRV-C in past few years may partly due to (1) clinical complications of HRV were generally mild during the course of infection and repeat infections can occur frequently with different HRV strains, thus less attention by the medical personnel and (2) the virus detection method previously utilized primer sets for 5'UTR amplification had limited sensitivity for distinguishing between HRV-C and HRV-A. Even though the large amount of sample size was utilized in this recent study, further investigations of HRV evolutionary history were still warranted because of the inadequate of sample during the early of 1990s and since the majority of the specimens were identified recently. Moreover, limited number of complete coding sequence and P1 sequence of HRV-C might hamper the analysis of selective pressure operating on HRV adaptive evolution. To better understand the dynamic relationship and host-virus interaction, further research using large number of sequences obtained from several geographic areas with an adequate period of time will be required.

Even HRV and HEV68 in VP1 gene particularly display high substitution rates and genetic heterogeneity was similar to those observed for the surface glycoproteins of the Flu [186, 187] and HIV-1 [188], genetic diversity of both of HEV68 and HRV do not undergo influenza-like antigenic drifts. As with other picornaviruses, the evolution may undergo random processing under strong purifying diversification with an island of adaptive

selected residues in structural region [77, 85, 100, 138, 189, 190]. This constrained mutation of HRV and HEV68 can be explained by the limited size and the genetic architecture of the viral genome which is overlap between structural and functional domains [85, 87, 191]. With regard to adaptive mutation of HEV68 under serological selection, the ω values of specific residues in the VP1 were determined. The result suggested that HEV68 tends to escape from stabilizing selection by positively mutating some residues in the antigenic epitope, in particular the residue located on the surface BC and DE loops, resulting in discrimination between the 3 distinct phylogenetic lineages. Differences in the classification of HEV68 have been reported by Rahamat-Langendoen et al [45]. Two distinct evolutionary lineages comprising old and new clusters were proposed based on phylogenetic analysis of the structural VP4/VP2 and VP1 and year of identification. The old cluster comprised HEV68 strains identified in the Netherlands in 2009 and 2010. The new cluster contained all 2010 strains and some of these strains had one amino acid deletion in the VP1. Upon combining these characteristics, the previously defined old and new groups could be reclassified within cluster 1 and 2, respectively. This study further suggested that sharing of genetic information inter and intra-species between some of their ancestors may be an important dynamic mechanism for HRV evolution as phylogenetic incompatibility in all HRV species was shown. Based on the phylogenetic relationship and compatibility analysis results, this study suggested the elementary units shaping the genetic diversity of HRV-C could be found in the nonstructural 2A and 3D genes. Genetic diversification of HEV68 may not be driven by recombination process since no recombination was detected when each gene of HEV68 was analyzed separately. However, due to the limit numbers of HEV68 sequence and evolutionary characteristic, the conclusion could not be drawn if this evolutionary process holds for HEV68.

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APPENDIX A

INNATE AND ADAPTIVE IMMUNE RESPONSE TO ENTEROVIRUS INFECTION

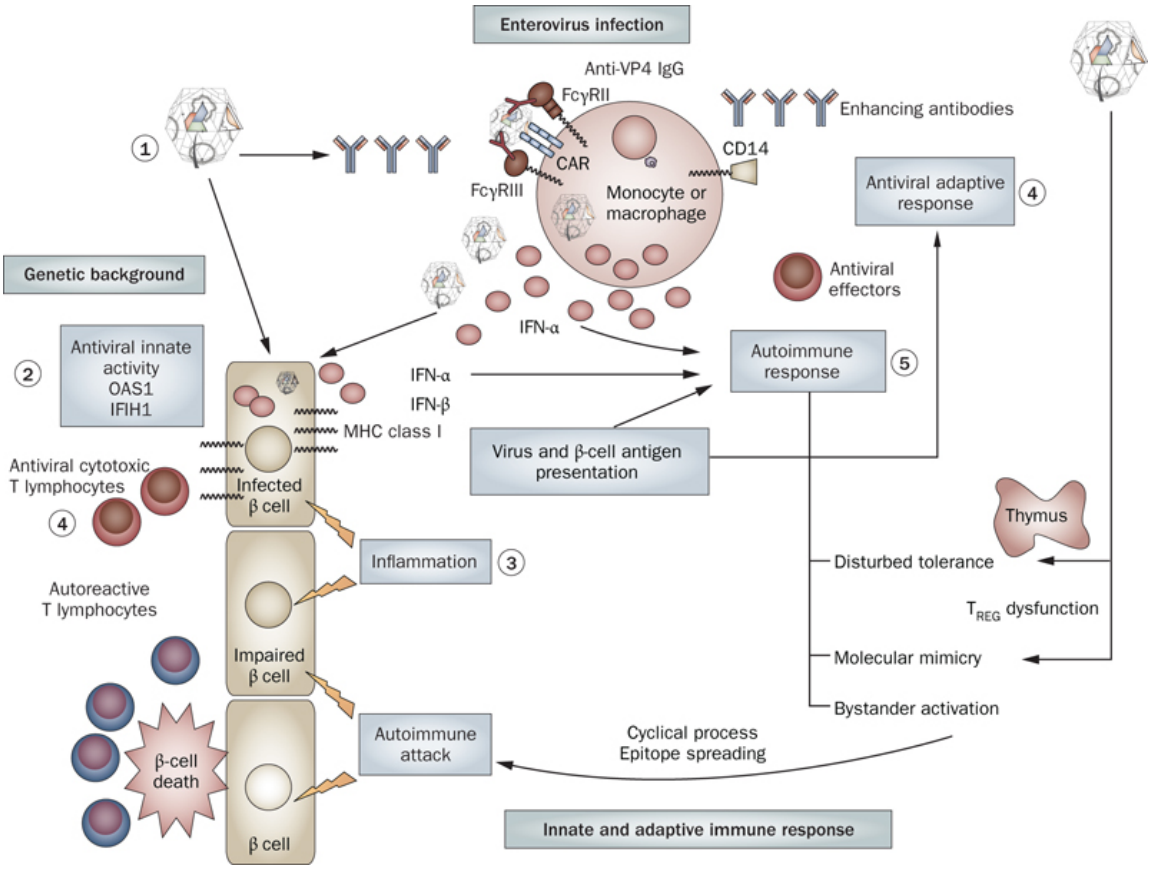

Figure S1. Enterovirus-induced pathogenesis [193]

APPENDIX B

MICRO RNA

The mechanism by which miRNAs regulate the targeted genes expressions is influenced by several parameters including the sequence complementarity between each other strand and multiplicity and secondary structures and locations of the target site. Perfect or near-perfect match of miRNA-mRNA efficiently leads to targeted genes cleavage and degradation. Instead, the translational efficiency is reduced by target gene silencing through the imperfect basepairing of miRNA-target mRNA.

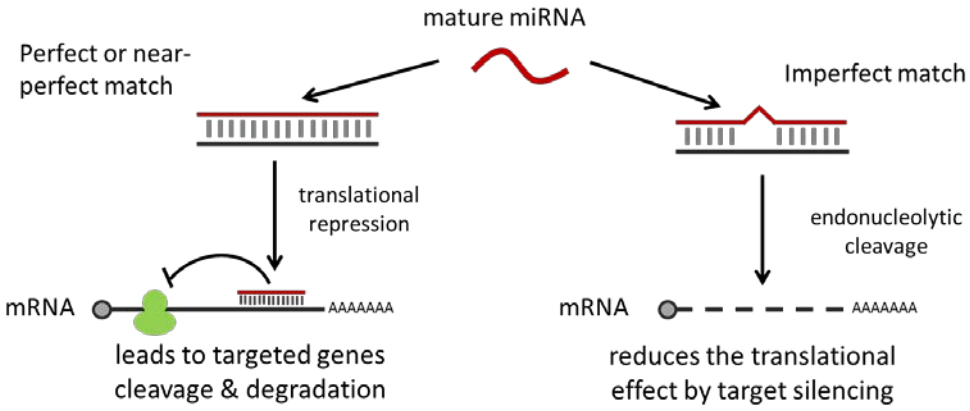

Fig S2. Quality of RISC-incorporated miRNA and target mRNA pairing

The most widely acceptable parameter used to identify the functional miRNA is the quality of miRNA-target pairing. Recently, mechanism for gene regulation by miRNA can be categorized to 2 types; cis-regulation which microRNA direct targeting the mRNA and regulating the expression of the target gene at post-transcriptional levels such as enhance mRNA degradation and inhibit translation and trans-regulation which miRNA may alter the transcription of other gene, levels of other mRNAs, or interactions among proteins, and thus microRNA may exert its functional effects (Fig S3). Specificity and quality of miRNA and target recognition relied on the complementarity of miRNA and target. Brennecke and colleagues suggested the 2 patterns of miRNA-target with different in ability to regulate gene expression;

(1) 5′ Dominant is the duplex structures rely on more than 7 base-pairing between 5′end of the miRNA seed region and target site. The 5′ dominant can be subdivided to 2 subgroups based on the base-pairing quality at the 3′ end of miRNA including 5′ canonical

duplex which require both of 5′ and 3′ strong base-pairing support and 5′seed′ site is the duplex which base pair well at the 5′ end of miRNAs with little or no 3′ basepairing. Based on the experimental results reported by several studies, 5′ canonical is the most currently identified pattern and has been shown to be more effective function than other pattern.

(2) 3′ compensatory site is a site with strong compensatory paring at the 3′ end of miRNA with weak 5′ basepairing.

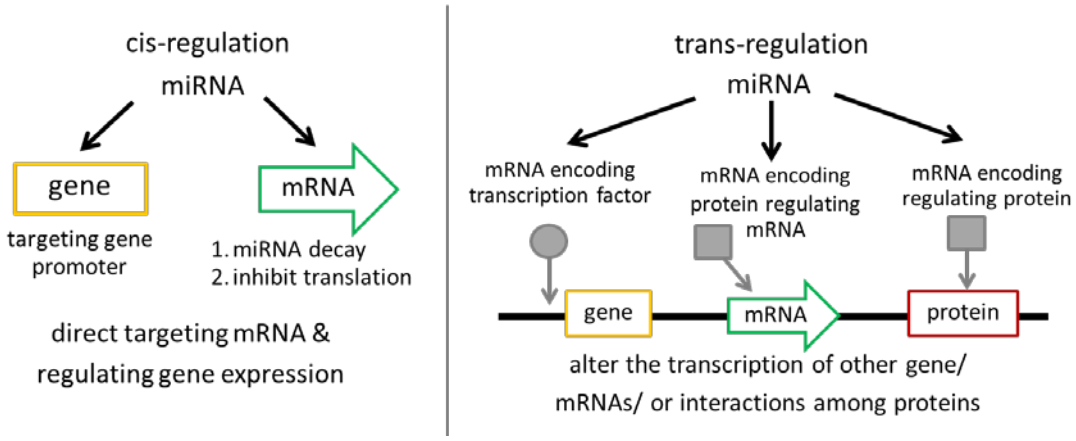

Figure S3. Potential regulation mechanism of miRNA

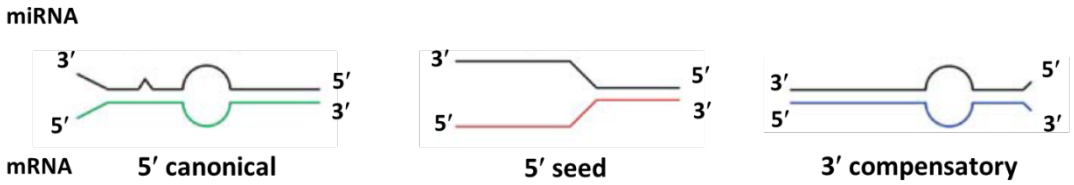

Figure S4. Schematic represents patterns of miRNA-target duplex structures.

APPENDIX C

PICORNAVIRUS CLASSIFICATION AND PHYLOGENETIC ANALYSIS

Definitions of a picornavirus genus and species

In order to classify a new picornavirus belongs to an existing genus or a new genus, the virus should have all characteristics match with the criteria described as following;

Species

Picornavirus species is a class of phylogenetically related serotypes or strains which would normally be expected to has (1) an identical genome architecture and function (2) significantly has high compatibility in replication process, translation, proteolytic processing, encapsidation and genetic recombination and (3) share a limited range of hosts and cellular receptors [194].

Genus

Some preliminary guidelines for a new picornavirus genus classification are described following; members of a genus should normally share (1) structurally and functionally homologous in the Leader protein, 2A protease, 2B and 3A polypeptides or (2) have homologous structure of an internal ribosome entry site (a new virus would be expected to has at least 1 criteria) and (3) should normally share phylogenetically related P1, P2 and P3 genome regions, each sharing more than 40% amino acid identity for P1 and P2 and more than 50% amino acid identity in P3.

APPENDIX D

PRIMER SEQUENCES AND PCR CONDITIONS

Table S3: Primer sequences for HRV-A sequencing

pair	Primer's name	Sequence (5'-3')	Position	region
1	HRV_A_F1486	TATGTCAATGCWGTNCCHATGGA	1486-1509	VP2
	HRV_A_R2159	TGGACTGCAANCCHAYATCCCA	2159-2138	VP3
	HRV_A_R2273	GAAGTATAAGAGYDGTYTGRTACCA	2273-2249	
2	HRV_A_F2014	GCGCTTTAGYTTYATGTTYTGTGG	2014-2037	
	HRV_A_R2782	ACTATTGTTATYTCWGARTCAAAYCT	2782-2757	VP1
	HRV_A_R2865	GTGCTCCTGGDGGNACASACAT	2865-2841	
3	HRV_A_F2697	GAAGGTTAGTCTTMARGARATGGC	2697-2719	
	HRV_A_R3426	CTTCAGTGTTTGTKCGRTADATGA	3426-3403	2A
	HRV_A_R3546	ATTCTGATTCYTGDATYTCAWACCA	3546-3522	
4	HRV_A_F3390	TCCAGTGATCTARTCATHTAYCGMACA	3390-3416	
	HRV_A_R3982	AGAGTTTCTCTTTNARRAAYCTCCA	3982-3957	2B
	HRV_A_R4038	CTGCATTACACATYTCWGTRAAYTT	4060-4038	2C
5	HRV_A_F4550	ACAGAGTGTNGTNATHATGGATGA	4550-4526	
	HRV_A_R5153	GTACACTCTGCTGGDAHNAYCCA	5153-5131	3A
	HRV_A_R5466	GCCTGGNTCAGMATGDGTDGG	5466-5446	3C
6	HRV_A_F5555	TTGTGAAATTAANTAGNAATGARAARTT	5555-5582	
	HRV_A_F5680	GTTGGTGATGYNGTNTCNTATGG	5680-5702	
	HRV_A_R6248	GAAACATADGGAWANCCTGCACT	6248-6226	3D
7	HRV_A_F6210	TTTGGATTTDAAYACHAGTGCWGG	6210-6233	
	HRV_A_R6659	AAGAAAADATNACATCATCHCCATA	6683-6659	
	HRV_A_R7114	CATACATGADGGNTTYTTNGTCCA	7114-7091	
8	HRV_A_F6577	GCTTTTGATTAYWCAAAYTATGAYGG	6577-6602	
	HRV_A_R7114	CATACATGAAGGDTTYTTNGTCCA	7137-7112	3'UTR
	HRV_A_R7137	CAAAGATAGNACRTGHTCNTGCAT	7114-7091	

Table S4: Primer sequences for HRV-B sequencing

pair	Primer's name	Sequence (5'-3')	Position	region
1	HRV_B_F749	GTTATTAAYTAYTAYAAGGATGCAGC	749-774	VP4
	HRV_B_F773	GCTGGTCAATCACTGTCAATGGA	766-789	
	HRV_B_R1512	TGTCATWGAATCMATDGGBACTGA	1512-1490	VP2
2	HRV_B_F1435	GTTTATTAATCTNAGRACMAAYAATAC	1435-1461	
	HRV_B_R2264	GCAAAATAAGDGAWGTYTGATACCA	2264-2240	VP3
	HRV_B_R2330	TCAACTTRAAATCTGRACARGCACT	2330-2304	
3	HRV_B_ F2109	CTAGGTACTCATGTDRTNTGGGAT	2084-2109	VP3
	HRV_B_ F2127	CATAGTAATGAMHATHCCNTGGAC	2127-2150	
	HRV_B_ R2700	GGACAGGTTGATYTTCCAATCATT	2700-2676	VP1
4	HRV_B_ F2379	AGTCATCGTTGABAAARYGAAACAG	2379-2503	VP1
	HRV_B_ R3063	TATTCTGAATGCHATRCTDCCCAT	3063-3040	
	HRV_B_ R3005	GCATCATCATGTGARTANCCATC	3005-2983	
5	HRV_B_ F2882	CAAAGTGCYTCWAAYCCHAGTGT	2882-2905	
	HRV_B_ R3544	TGCCGGNCCHACHCCTTTCAT	3544-3523	2A
	HRV_B_ R3663	TCGTATGTCHGCRAARCANACATA	3663-3639	2B

pair	Primer's name	Sequence (5'-3')	Position	region
6	HRV_B_ F3484	CCCACCAACATNTGGATHGAAGG	3484-3507	2A
	HRV_B_ R4254	CTTTTGCGACATYTGYTCYAACCA	4254-4231	2C
	HRV_B_ R4367	TAATACACANACTGGHTCNGTTCT	4367-4343	
7	HRV_B_ F3991	GATGGATGGTTYARRAARTTYAATGA	3991-4017	
	HRV_B_ F4026	TGCTGCAAARGGHYTRGARTGGAT	4026-4050	
	HRV_B_ R4866	TACCAAGCTAABDGCTTTDCCACA	4866-4842	
8	HRV_B_ F4543	GATATAAGYATDTTYTGYCARATGGT	4543-4568	
	HRV_B_ R5237	TATGGWCCYTGDGTYTGDGCAAA	5237-5215	3B
	HRV_B_ R5318	GCAAAYTCAGTRTTDGGNCCTTG	5318-5295	3C
9	HRV_B_ F5770	GGTATTCATGTBGGHGGHAATGG	5770-5792	
	HRV_B_ R6412	TTCATTCTCATRTTNACWGARTCATT	6412-6388	3D
	HRV_B_ R6563	GAGGCATCAAAATTTGARTARTCAAA	6576-6553	
10	HRV_B_ F6302	CCTCTAGTTACHTAYATHAARGATGA	6302-6327	
	HRV_B_ R7032	TATATCTTDCATTGGCATNACTGG	7032-7008	
	HRV_B_ R7116	TCTCCTGARTGCCADGCYAACAT	7116-7093	3'UTR

Table S5: Primer sequences for HRV-C sequencing

pair	Primer's name	Sequence (5'-3')	Position	region
1	HRV_C_F857	CCGATAGGCTNAARCAAATYACTAT	844-869	VP2
	HRV_C_ F979	GATAAACCYACNCAYCCNGAAAC	962-989	
	HRV_C_ R1481	GTTGTGCCNNARCATRYTRTCCAT	1447-1470	
2	HRV_C_ F1164	TAATGCCACMAAATTYMAYAGTGG	1152-1175	
	HRV_C_ F1389	CACCAGCTNATMAAYTTGMGGAC	1377-1400	
	HRV_C_ R2010	CAGTACTRAARCTRTCACANACACA	1951-1975	VP3
3	HRV_C_ F2022	GATAGCMTACACMCCNCCAGG	1986-2006	
	HRV_C_ F2291	ATGNTRCGNGACACACCNATGAT	2257-2279	
	HRV_C_ R3212	GTTCTGTRTGNACAWANAKRTCACT	3133-3158	VP1
4	HRV_C_ F3630	ACACAAYTAGGNARYGCNTTTGG	3574-3596	2B
	HRV_C_ R4433	GGGTTYTGRCCNANATCATCCAT	4360-4382	2C
	HRV_C_ R4485	CAGTGNTNGANACCATYTGACA	4408-4429	
5	HRV_C_ F4401	CARGANGTGGTRATNATGGATGAT	4345-4368	3A
	HRV_C_ R5300	GCATGTGTKGGTANNACWGCACA	6240-6217	3B
	HRV_C_ R5441	CTTCTTATRTCYCTRAACTTYTCATT	5381-5356	3C
6	HRV_C_ F5027	CARTATGCTNTNAATTGYNTNGGTAG	4969-4994	3A
	HRV_C_ R5834	CAGGAAACAYATCRTRGAANACACT	5749-5774	3D
	HRV_C_ R6019	CCATGGWNATNGGNTCWGGGTT	5938-5959	
7	HRV_C_ F5596	CCACNMAACCAGGNCANTGTGG	5539-5561	3D
	HRV_C_ R6402	CAACGWAGCATNNATNYGTGACCA	6319-6342	
	HRV_C_ R6446	CTNCCATCATARTTKGTNTANTCAAA	6361-6387	
8	HRV_C_ F6312	ACTGSYTTYCATGCMAAYCCAGG	6252-6275	
	HRV_C_ R7009	TCTYTRGTCCAYCTGATYGATTCA	6925-6947	
	HRV_C_ R7116	CCATTYANATGAANTNNTCNATCCA	7030-7053	3'UTR

Table S6: Primer sequences for amplification and sequencing of HRV and HEV68.

Type of study	Primer name	Region	Sequence (5′-3′)	Position
HRV/HEV screening	F484	5′UTR	CGGCCCCTGAATGYGGCTAA	447-466[a]
	F578	5′UTR	CTACTTTGGGTGTCCGTGTTTC	540-561[a]
	R1126	VP2	ATCHGGHARYTTCCAMCACCA	1077-1057[a]
HEV68 screening	2325-fwn	VP1	GGRTTCATAGCAGCAAAAGATGA	2323–2345[b]
	2547-fwni	VP1	AAGCCATACAAACTCGCACRGT	2546–2567[b]
	3121-rvni	VP1	TAGGYTTCATGTAAACCCTRACRGT	3154–3130[b]

F = Forward or sense primer/ R = Reverse or antisense primer

a = Primer positions are given based on multiple sequence alignments of HRV available at the GenBank Database and numbered according to HRV89 (A10937).

b = Primer positions are based on the nucleotide positions of the HEV68 prototype, Fermon strain (AY426531).

Degenerate bases will be assigned using IUPAC ambiguity codes: M (AC), R (AG), Y (CT), W (AT), S (CG), K (GT), V (ACG), H (ACT), D (AGT), B (CGT), and N (ATCG).

Table S7: General condition for semi-nested PCR amplification

PCR reaction	semi-nested PCR				Number of amplification cycle
	1st PCR		2nd PCR		
	Temperature	Duration	Temperature	Duration	
Initial denaturation	94°C	3 min	94°C	3 min	1
Denaturation	94°C	40 sec	94°C	40 sec	35-40
Annealing*	50-52 °C	40 sec	52-55°C	40 sec	
Extension	72°C	2 min	72°C	2 min	
Final Extension	72°C	7 min	72°C	7 min	1
Hold	25 °C	5 min	25 °C	5 min	1

Table S8: Primer sequences for amplification and sequencing of RSV.

Type of study	Primer name	Sequence (5′-3′)
RSV-A screening	RSVA_227F1	TACAAGATGCAACAARCCAGATCA
	RSVA_234F2	TGCAACAARCCAGATCAAGAACA
	RSVA_1072R	CTAACTGCACTGCATGTTGATTGA
RSV-B screening	RSVB_225F1	AGTTCAAACAATAAAAAACCACACTG
	RSVB_225F2	ACCTTACTCAAGTCYCACCAGAAA
	RSVB_1009R	TGCATTAATAGCAAGAGTTAGGAAG

APPENDIX E

Table S9: Seasonality of HRV in tropical and temperate climate countries.

Climate	Country	Study period	Diseases	Study design	Detection method	Seasonality of HRV infection
Tropical	Sao Paulo, Brazil [2]	Jun 2001 to Sep 2003	ILI	Retrospective study of respiratory virus in 420 subjects (health care workers, practitioners and outpatients)	RT-PCR hybridization assays	Year round with high detection rate in winter months (July-Sep)
	Salvador, Brazil [152]	Sep 2003 to May 2005	CAP	Retrospective study of viral and bacterial infections in 184 children aged <5yrs	PCR	Mainly detected in winter (July-Sep); correlated with RH without statistical significance
	Sa Kaeo, Thailand [195]	Sep 2003 to Aug 2005	ILI and LRTI	Retrospective study conducted among 1919 hospitalized patients and 280 controls of all ages	RT-PCR and semi-nested PCR	Year round with small peak during summer months (Apr-May)
Temperate	Mainz, Germany [156]	2001 to 2006	ARTI	Retrospective study of 3044 children hospitalized with ARIs	Multiplex RT-PCR-ELISA	Inverse correlated with temp but direct correlated with RH
	New York, United States [110]	Oct 2001 to Sep 2003	ARTI or fever	Prospective population-based surveillance among children <5yrs	Real-time RT-PCR	More HRV strains detected in Mar-Apr and Sep-Oct
	Edinburgh, United Kingdom [105]	Sep 2006 to Sep 2007	ARTI and non-relevant	Comprehensive surveillance among 1540 children <5yrs (2787 samples)	PCR	HRV-C infections occurred year round
	Nanjing, China [124]	Dec 2006 to Nov 2007	ARTI	Surveillance study in 406 samples from children <14yrs	PCR	Predominately in winter and spring

CAP = Community acquired pneumonia/ARTI = Acute respiratory tract infection/ RT-PCR =Reverse transcriptase polymerase chain reaction/ ELISA = Enzyme-linked immuno sorbent assay

APPENDIX F

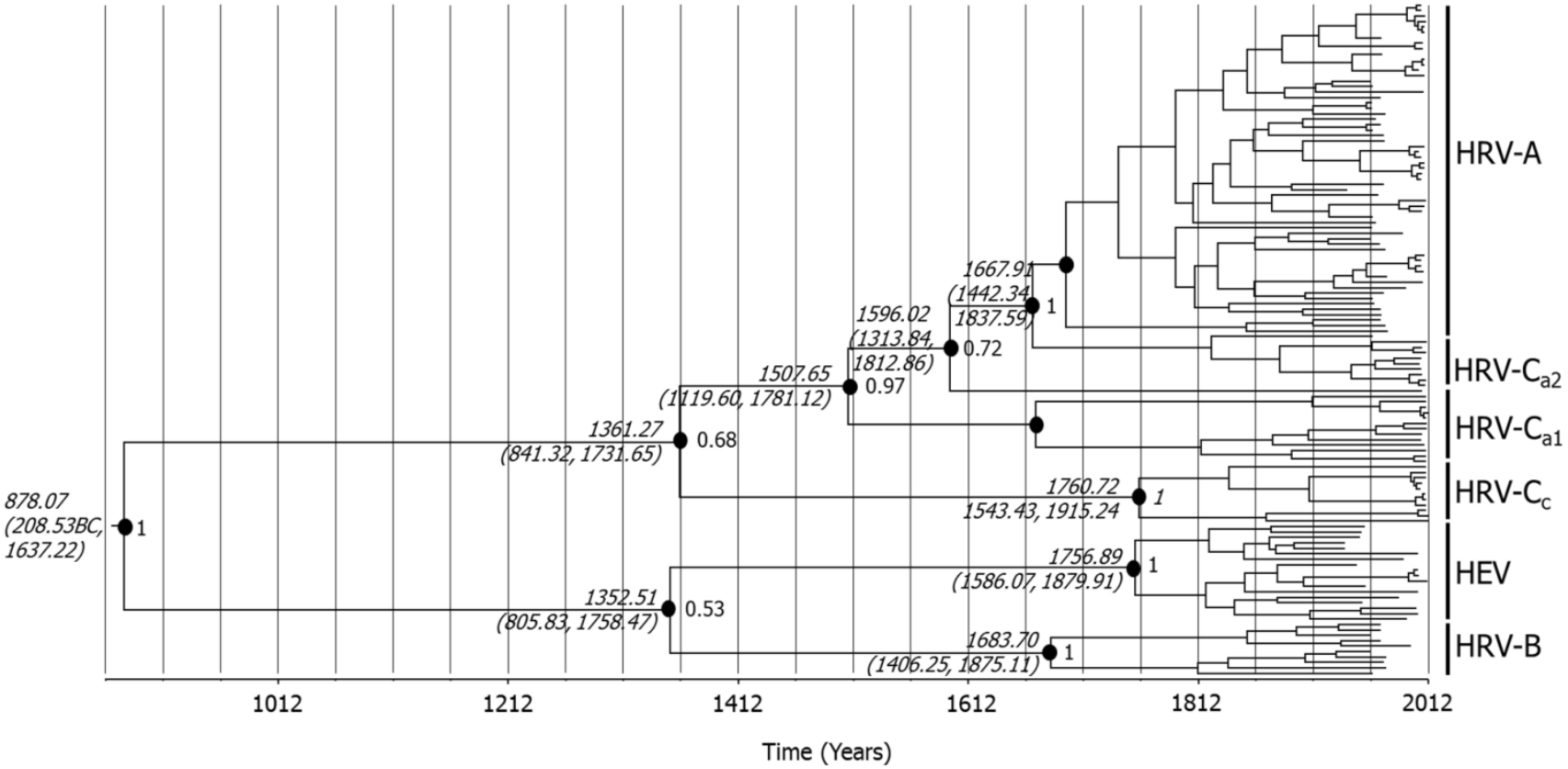

Figure S5 (32 cont.): Dated phylogeny inferred with the HRV and HEV 5'UTR through Bayesian analysis implementing exponential relaxed molecular clock model

BIOGRAPHY

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1. Linsuwanon P, Auksornkitti V, Suwannakarn K, et al. Epidemiology, seasonal periodicity and evolutionary characteristics of human rhinovirus in Thailand. *(pending)*
2. Puenpa J, Chieochansin T, Linsuwanon P, Korkong S, et al. Large Scale Outbreak of Coxsackievirus A6, Thailand. Emerg Infect Dis.2012
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