Epidemiology, Molecular Genetic, and Evolutionary Analysis of Human Rotavirus A Infection in Thailand

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ระบาดวิทยาอณูพันธุศาสตร์และการวิเคราะห์วิวัฒนาการของการติดเชื้อโรคโรตาไวรัสของมนุษย์ในประเทศไทย

น.ส.ฟาจาร์ บุดี เลสตารี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ฟาจาร์ บุตี เลสตารี : ระบาดวิทยาอณูพันธุศาสตร์และการวิเคราะห์วิวัฒนาการของการติดเชื้อโรคโรตาไวรัสของมนุษย์ในประเทศไทย. (Epidemiology, Molecular Genetic, and Evolutionary Analysis of HumanRotavirus A Infection in Thailand) อ.ที่ปรึกษาหลัก : Prof.ยง ภู่วรวรรณM.D., อ.ที่ปรึกษาร่วม : ดร.สมพงษ์ วงษ์พันสวัสดิ์Ph.D.

การติดเชื้อไวรัสโรต้า เอ ยังคงเป็นหนึ่งในสาเหตุสำคัญของการเกิดโรคอุจจาระร่วงที่มีสาเหตุจากเชื้อไวรัสในเด็กทั่วโลก แม้ว่าความสำเร็จของการใช้วัคซีนไวรัสโรต้า เอ ชนิดหยอด 3 ครั้ง อย่าง RotaTeq สามารถลดอัตราการเกิดโรคและลดความรุนแรงของโรคในการเข้ารับการรักษาในโรงพยาบาลได้อย่างมีนัยสำคัญ สำหรับการสร้างเสริมภูมิคุ้มกันแห่งชาติต่อไวรัสโรต้าเพิ่งเริ่มต้นในประเทศไทยเมื่อปี พ.ศ. 2563 เพราะเหตุนี้จึงมีความเป็นไปได้ว่าการฉีดวัคซีนป้องกันไวรัสโรต้าในกลุ่มเด็กอาจยังไม่ได้มีการบันทึกข้อมูลอย่างจริงจัง. ในส่วนแรกของการศึกษานี้เป็นการสรุปข้อมูลเกี่ยวกับไวรัสวิทยา, ภาระโรค, ความชุก, การแพร่กระจายของสายพันธุ์และฤดูกาลการระบาดของเชื้อไวรัสโรต้า และสถานการณ์ปัจจุบันของการได้รับวัคซีนป้องกันไวรัสโรต้าในประเทศภูมิภาคเอเชียตะวันออกเฉียงใต้ (กัมพูชา, อินโดนิเซีย, สาธารณรัฐประชาธิปไตยประชาชนลาว, มาเลเซีย, พม่า, ฟิลิปปินส์, สิงคโปร์, ไทย, และเวียดนาม) ตั้งแต่ปี พ.ศ. 2551 ถึง พ.ศ. 2561 ตามที่ข้อมูลการเฝ้าระวังของประเทศในภูมิภาคเอเชียตะวันออกเฉียงใต้ พบว่าร้อยละ 40.78 ของโรคอุจจาระร่วงในเด็กมีสาเหตุมาจากการติดเชื้อไวรัสโรต้า อีกทั้งอัตราการเสียชีวิตมีค่าแปรผกผันกับสถานะทางเศรษฐกิจและสังคม โดยสายพันธุ์ที่พบมากที่สุดของเชื้อไวรัสโรต้ามีการเปลี่ยนแปลงจากสายพันธุ์ G1P[8] และ G2P[4] กลายเป็นสายพันธุ์ที่หายากและแตกต่างไปจากเดิมนั่นคือสายพันธุ์ G3P[8], G8P[8], และ G9P[8] แม้ว่าสายพันธุ์ที่พบบ่อยจะมีการเปลี่ยนแปลง แต่ฤดูกาลที่พบการติดเชื้อไวรัสโรต้ายังคงไม่เปลี่ยน อย่างไรก็ตาม วัคซีนป้องกันไวรัสโรต้ายังคงมีราคาสูงในประเทศภูมิภาคเอเชียตะวันออกเฉียงใต้เนื่องจากอัตราส่วนระหว่างการสูญเสียปีสุขภาวะ (DALY) กับผลิตภัณฑ์มวลรวมในประเทศ (GDP) ต่อคนน้อยกว่าหนึ่ง

ในส่วนที่สองของการศึกษานี้ ทางผู้วิจัยได้ทำการศึกษาในช่วงสถานการณ์การแพร่ระบาดของเชื้อไวรัสโคโรนาในปี พ.ศ. 2563 - 2564 โดยมีสิ่งส่งตรวจของผู้ป่วยโรคอุจจาระร่วงทั้งหมด 257 ตัวอย่างจากโรงพยาบาล 4 แห่งในประเทศไทย ผลการศึกษาพบว่า มีเพียง 25 ตัวอย่าง (ร้อยละ 9.7) เท่านั้นที่ให้ผลบวกต่อเชื้อไวรัสโรต้า เอ และพบว่าสายพันธุ์ G3P[8] เป็นสายพันธุ์ที่พบบ่อย และมี 8 ตัวอย่างจากตัวอย่างที่ให้ผลบวกทั้งหมด พบว่ามีความหลากหลายของเชื้อไวรัสโรต้าจากการวิเคราะห์ข้อมูลลำดับพันธุกรรมในส่วนของยีน VP7 และ VP4 โดยมี 2 ตัวอย่างที่ลำดับพันธุกรรมเหมือนกับสายพันธุ์วัคซีน ของ RotaTeq เมื่อวิเคราะห์กลุ่มลำดับพันธุกรรมทั้งหมดของเชื้อไวรัสโรต้าจากตัวอย่างหนึ่ง (B8019) พบว่าสอดคล้องกับ G1P[8] ของสายพันธุ์ดั้งต้นของวัคซีน เมื่อวิเคราะห์อีกตัวอย่างหนึ่ง (B7711) พบว่าประกอบด้วย G1, G2, G3, G4, P[5], และ P[8] เหมือนกับสายพันธุ์ในวัคซีน เช่นเดียวกับ G3P[4] สายพันธุ์ดั้งเดิมของเชื้อไวรัสโรต้า เอ ที่พบในม้าด้วย โดยก่อนหน้านี้ไม่มีการรายงานของผู้ป่วยโรคอุจจาระร่วงจากการติดเชื้อไวรัสโรต้า เอ ภายหลังได้รับวัคซีน RotaTeq ในประเทศไทย ผลการศึกษานี้จึงแสดงให้เห็นว่า จำเป็นต้องตระหนักให้มากขึ้นถึงโรคอุจจาระร่วงที่มีสาเหตุมาจากไวรัสโรต้า เอ ภายหลังจากการได้รับวัคซีนและการศึกษานี้ยังแสดงให้เห็นถึงโอกาสของการเกิดการติดเชื้อร่วมกับสายพันธุ์ดั้งเดิมของเชื้อไวรัสโรต้า เอ ภายหลังการได้รับวัคซีนไม่นาน

แม้ว่าวัคซีนไวรัสโรต้าจะมีการนำมาใช้ในหลายประเทศทั่วโลกและมีประสิทธิภาพในการลดอุบัติการณ์โดยรวมของการติดเชื้อไวรัสโรต้า แต่เชื้อไวรัสนี้ยังคงเป็นสาเหตุหลักของการเกิดโรคอุจจาระร่วงในประเทศด้อยพัฒนารวมถึงในประเทศไทย นอกจากนี้ความหลากหลายของสายพันธุ์ของเชื้อไวรัสโรต้า เอ รวมถึงการเพิ่มขึ้นของสายพันธุ์ที่พบได้บ่อยอย่าง G3 (วิเคราะห์จากยีนส่วน VP7) ซึ่งพบได้ทั้งในคนและสัตว์ ในการศึกษานี้ได้มีการเฝ้าระวังการแพร่ระบาดในกรุงเทพมหานคร โดยพบสายพันธุ์ G3 ในผู้ป่วยที่มีอาการอุจจาระร่วงที่ไม่เหมือนสายพันธุ์วัคซีนและมีการเปลี่ยนแปลงที่หลากหลายของลำดับพันธุกรรม ในการศึกษาส่วนที่สามของงานวิจัยนี้ ทางผู้วิจัยได้วิเคราะห์จีโนมทั้งหมด 7 ตัวอย่างของสายพันธุ์ G3 พบว่ามีการแลกเปลี่ยนจีโนไทป์ โดยมีการจับคู่กับ P[4], P[6], P[9], และ P[10] (วิเคราะห์สายพันธุ์จาก VP4) ที่น่าสนใจคือ ทางผู้วิจัยพบสายพันธุ์ไวรัสโรต้าที่มีความใกล้เคียงกับสายพันธุ์ที่พบในค้างคาวโดยการวิเคราะห์จากกลุ่มลำดับพันธุกรรม ซึ่งพบว่าเป็น G3-P[10]-I3-R3-C3-M3-A9-N3-T3-E3-H6 และไม่มีการรายงานมาก่อนหน้านี้ เมื่อวิเคราะห์ตำแหน่งกรดอะมิโนของสายพันธุ์ G3 พบว่ามีความแตกต่างกับตำแหน่ง antigenic epitopes ในส่วนของยีน VP7 ของสายพันธุ์วัคซีน G3 แม้ว่าจะไม่ใช่เรื่องผิดปกติที่จีโนมของไวรัสโรต้า เอ จะมีการจัดกลุ่มใหม่และก่อให้เกิดสายพันธุ์ใหม่ แต่สายพันธุ์ G3 ที่มีการเปลี่ยนแปลง ทางผู้วิจัยแสดงให้เห็นว่ามีโอกาสที่จะเกิดการติดต่อจากสัตว์สู่คนของเชื้อไวรัสโรต้ารวมถึงในกรุงเทพมหานครด้วย

ในส่วนที่สี่ของการศึกษานี้เป็นการวิเคราะห์การเกิดโรคติดต่อระหว่างคนและสัตว์จากเชื้อไวรัส เนื่องจากการติดเชื้อไวรัสโรต้าเป็นสาเหตุของการเกิดโรคอุจจาระร่วงในสัตว์หลายชนิด ในการศึกษานี้พบว่าแมวเพศเมียสายพันธุ์วิเชียรมาศ เลี้ยงในอาคาร มีอาการถ่ายเป็นมูกเลือดและผลการทดสอบพบว่าให้ผลบวกต่อเชื้อไวรัสโรต้าด้วยวิธี real-time reverse-transcription polymerase chain reaction (RT-PCR) ต่อมาได้ทำ conventional RT-PCR และวิเคราะห์ลำดับนิวคลีโอไทด์พบว่ามีความใกล้เคียงกับเชื้อไวรัสโรต้าสายพันธุ์ G3P[9] และจำแนกกลุ่มจีโนมได้เป็น G3-P[9]-I2-R2-C2-M2-A3-N2-T3-E3-H3 เมื่อทำ phylogenetic analysis ของยีนส่วน VP4, VP7, NSP1, NSP3, NSP4, และ NSP5 พบว่ามีความใกล้เคียงกับเชื้อไวรัสโรต้าที่พบในคนและแมวในขณะที่ยีน VP1, VP2, VP3, VP6, และ NSP2 มีความใกล้เคียงกับเชื้อไวรัสโรต้าที่พบในวัว แม้ว่าสายพันธุ์ G3P[9] นี้ จะมีการรายงานมาก่อนหน้านี้ในประเทศเกาหลี โดยพบการติดเชื้อในเด็กผู้หญิงอายุ 9 ปี (สายพันธุ์ CAU-12-2-51) เมื่อ 10 ปีที่แล้ว แต่ยังไม่เคยมีข้อมูลในประเทศไทยและยังคงเป็นสิ่งที่น่าสงสัย

โดยสรุปงานวิจัยนี้ เป็นการเพิ่มเติมข้อมูลของความสำคัญในการใช้วัคซีนในการป้องกันการติดเชื้อไวรัสซึ่งถือเป็นสิ่งสำคัญในการพัฒนาเพื่อก่อให้เกิดความเข้าใจเกี่ยวกับชีววิทยาที่เกี่ยวข้องกับการเกิดโรคในประเทศไทยและอีกหลายพื้นที่ทั่วโลก

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KEYWORD: Rotavirus, Diarrhea, Vaccine, Genome constellation, Zoonosis, Human, Animal

Fajar Budi Lestari : Epidemiology, Molecular Genetic, and Evolutionary Analysis of HumanRotavirus A Infection in Thailand. Advisor: Prof. YONG POOVORAWAN, M.D. Co-advisor: Sompong Vongpunsawad, Ph.D.

Rotavirus A (RVA) infection remains one of the major causes of viral diarrhea in young children worldwide. Despite the success of RVA vaccines including RotaTeq in significantly reducing morbidity and disease severity associated with hospitalization, national immunization against RVA have only just begun in Thailand in 2020. Consequently, possible RV vaccine shedding among pediatric vaccine recipients has not been rigorously documented here.The first part of my study was summarize the virology, disease burden, prevalence, distribution of genotypes and seasonality of RVs, and the current status of RV vaccination in Southeast Asia (Cambodia, Indonesia, Lao People's Democratic Republic, Malaysia, Myanmar, Philippines, Singapore, Thailand, and Vietnam) from 2008 to 2018. According to the RV surveillance data for Southeast Asia, 40.78% of all diarrheal disease in children were caused by RV infection. Mortality was inversely related to socioeconomic status. The most predominant genotype distribution of RV changed from G1P[8] and G2P[4] into the rare and unusual genotypes G3P[8], G8P[8], and G9P[8]. Although the predominat strain has changed, but the seasonality of RV infection remains unchanged. Rotavirus vaccine is highly cost effective in Southeast Asia countries because the ratio between cost per disability-adjusted life years (DALY) averted and gross domestic product (GDP) per capita is less than one.

For the second part of my project done during the coronavirus pandemic of 2020 and 2021, I received 257 diarrhea samples from four sentinel hospitals in Thailand. Only 25 samples (9.7%) tested positive for RVA and G3P[8] was the predominant genotype. Eight samples contained multiple RVA strains based on detailed sequence analysis of the VP7 and VP4 genes, of which two samples possessed RVA with genetic similarity to the vaccine strains in RotaTeq. Genome constellation of one sample (B8019) was consistent with G1P[8] vaccine strain reassortant. Another sample (B7711) contained G1, G2, G3, G4, P[5], and P[8] vaccine strains, as well as equine-G3P[4] wildtype RVA. Neither report of diarrhea from RVA infection after RotaTeq vaccination nor simultaneous shedding of vaccine-derived and wildtype RV infection has previously been described in Thailand. These results suggest the need for increased awareness of RVA-associated diarrhea following routine vaccination and demonstrate evidence of possible co-infection with wild-type RVA shortly after vaccination.

Although rotavirus vaccines are available in many parts of the world and are effective in reducing the overall incidence of rotavirus infection, it remains a major cause of diarrhea in less-developed countries including Thailand. Among various RVA strains, the increasingly common genotype G3 (defined by the VP7 gene) has been identified in both humans and animals. Our previous epidemiological surveillance in Bangkok found several unusual non-vaccine-like G3 strains in patients with diarrhea. For the third part of my study, I sequenced and characterized the genomes of seven of these G3 strains, which formed combinations with genotypes P[4], P[6], P[9], and P[10] (defined by the VP4 gene). Interestingly, I identified a bat-like RVA strain with the genome constellation G3-P[10]-I3-R3-C3-M3-A9-N3-T3-E3-H6, which has not been previously reported in the literature. The amino acid residues deduced from the nucleotide sequences of our G3 strains differed at the antigenic epitopes to those of the VP7 capsid protein of the G3 strain in RotaTeq vaccine. Although it is not unusual for the segmented genomes of RVA to reassort and give rise to emerging novel strains, the atypical G3 strains I identified suggest possible animal-to-human RVA zoonotic spillover even in Bangkok.

The fourth part of my project examined viral zoonosis because rotavirus infection can cause diarrhea in many animal species. A 2 year-old indoor female Siamese cat was ill with a mucus-bloody diarrhea and tested positive for rotavirus by real-time reverse-transcription polymerase chain reaction (RT-PCR). Subsequent conventional RT-PCR and nucleotide sequence analysis revealed a rotavirus G3P[9] genotype with the genome constellation G3-P[9]-I2-R2-C2-M2-A3-N2-T3-E3-H3. From phylogenetic analysis, the VP4, VP7, NSP1, NSP3, NSP4, and NSP5 genes were closely related to human/feline-like rotavirus, while VP1, VP2, VP3, VP6, and NSP2 genes were genetically closest to human bovine-like rotavirus. Although this G3P[9] strain was previously reported in Korea, which infected a 9 year-old girl (strain CAU-12-2-51) a decade ago, it has never been documented in Thailand and its emergence is enigmatic.

In summary, my thesis research projects collectively advances our knowledge of an important vaccine-preventable virus with a major impact in improving the understanding of disease biology in Thailand and elsewhere around the world.

Field of Study:	Biomedical Sciences	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....
		Co-advisor's Signature .....

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Fajar Budi Lestari

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CHAPTER I

INTRODUCTION

Background and Rationale

Viral intestinal infections are the most common cause of acute infectious diarrhea in the pediatric group and accounts for approximately 70% of episodes of acute infectious diarrhea in children [1]. Rotavirus, norovirus, adenovirus, and astrovirus are the recognized viral causes of pediatric gastroenteritis [2]. Rotavirus (RV) constitutes 1 of the 13 diarrhea etiologic agents measured in the 2016 Global Burden of Disease Study [3]. RV infections were responsible for approximately 128,515 deaths annually among children younger than 5 years [3, 4]. It is the most common cause of vaccine-preventable severe diarrhea [5].

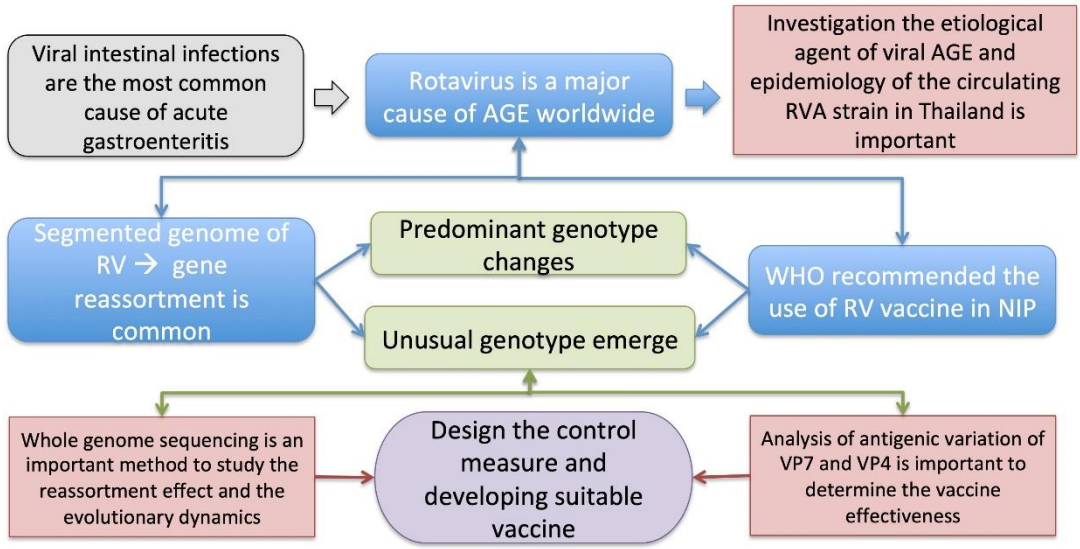
The rotavirus genome consists of 11 segments of double-stranded ribonucleic acid (RNA), with every segment coding for a viral protein. Because of its segmented genome, gene reassortment is common [6]. Reassortment resulted in the presence of a novel genotype that represents an important problem for vaccines. RV surveillance has become more important for monitoring changes in genotype distribution and vaccine effectiveness [7]. This study will focus on the molecular epidemiology of RV strains circulating in Thailand and will conduct molecular RV screening based on specific TaqMan probe real time reverse transcriptase polymerase chain reaction (RT-PCR) targeting the non-structural proteins 3 (NSP3) gene. Multiplex RT-PCR has been performed for RVA genotyping. Amino acid substitution and structural conformation study will

investigate the amino acid mutation and structure of the antigenic site of circulating RVA strain compared to the vaccine strain. Deep investigation of the unusual circulating strain will be conducted by whole genome sequencing (WGS).

Objectives

1. To investigate the etiological agent of viral acute gastroenteritis (AGE) and epidemiology of the circulating RVA strain in Thailand in 2020-2021.
2. To investigate the evolutionary pattern of unusual circulating RVA strain by whole genome sequencing.
3. To investigate the impact of vaccine introduction in the circulating RVA strain in Thailand

Experimental Design



Expected Benefits

The real-time RT-PCR assay based on probe targeted NSP3 will be useful for screening RVA infections, specifically in a routine diagnostic laboratory. Conventional multiplex RT-PCR can detect multiple strain infection of RVA. This study is expected to find how amino acid substitution changes the structure of the antigenic site in the viral protein 7 (VP7) and VP4 protein of RVA strains may affect the successful implementation of RV vaccines. Possible antigenic differences between circulating RVA strains after vaccine introduction in Thailand and RVA vaccines, Rotarix and RotaTeq. The differences might result in selection for strains that escape the RVA neutralizing-antibody pressure induced by vaccines. This study explored the origin of unusual RVA strains circulated in Thailand. Continuing surveillance of rotavirus genotypes circulating before and after the introduction resulted in a better understanding of the genetic variability and evolutionary dynamics of the new circulating RVA. It may contribute to determining future efficacy and the need to update vaccine components.

CHAPTER II

LITERATURE REVIEW

Background

Rotavirus (RV) History

RV was first identified in cattle in 1969 [8]. The virus appeared like those that cause diarrhea in mice [9], calves [10], and a virus identified from a rectal swab of a healthy monkey [11]. In May 1973, Bishop, Davidson, Holmes, and Ruckexamined ultrathin sections of duodenal mucosa from children with AGE by electron microscopy (EM), and found abundant viral particles in the epithelial cell linings of the upper villous surface which were similar in appearance to the RVs discovered in animals before [12]. EM also revealed 70-nm particles in negatively stained fecal extracts [13]. The viral particle was initially identified by several names including reovirus-like, orbivirus-like, duovirus, infantile gastroenteritis virus, or a "new" virus. The wheel-like structure observed on EM eventually led to the naming consensus of Rotavirus (*rota* is Latin for wheel) [14]. RVs have now been shown to be a cause of diarrhea in the young of many mammalian and avian species [15].

RV Morphology

RVs are 70-nm, non-enveloped RNA viruses belonging to the family *Reoviridae*. The RV genome consists of 11 segments of double-stranded RNA (dsRNA) surrounded by a triple-layered capsid (Figure 1). Each genomic fragment encodes a protein of distinct functions. The outer layer

proteins (VP4 and VP7) mediate attachment and penetration; the inner layer is composed of VP2 protein and encloses the viral genome and the minor protein VP1, the viral RNA-dependent RNA polymerase, and VP3, the viral capping enzyme. The middle layer is composed of VP6, which interacts with and stabilizes the inner and outer layers [16]. VP6 defines species/group and subgroup specificities [17-19]. Except for segment 11, all RNA segments are monocistronic, encoding either structural viral proteins (VP1 to VP4, VP6, and VP7) or non-structural proteins (NSP1 to NSP5). Genome segment 11 encodes two proteins: NSP5 and NSP6 [16]. RVs can be differentiated by a dual classification system, based on the two outer capsid proteins, VP7 and VP4, that determine the G (VP7, glycoprotein) and P (VP4, protease-sensitive) genotypes [20]. Based on the antigenic properties of VP6, RVs are classified into 9 species designated A-D and F-J, and 2 putative species K and L [21, 22]. Recently, 41 G, 57 P, 31 I, 27 R, 23 C, 23 M, 38 A, 27 N, 27 T, 31 E, and 27 H genotypes among the RVA have been identified in human and animal species worldwide [23].

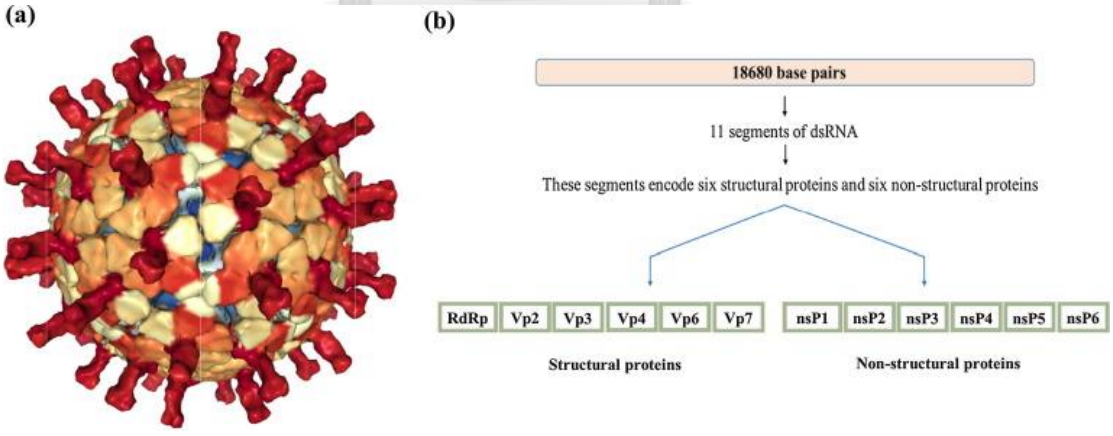


Figure 1. Rotavirus genome structure [24]

A whole genome-based genotyping system was recently proposed for RV Group A (RVA) based on the genotype assignment of all 11 gene segments [25]. The genome constellation of individual RV strains is given the complete descriptor of Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx to identify the genotypes of the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 encoding RNA segments, respectively. Most strains demonstrate either a Wa-like (G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1), DS-1 (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2), or AU-1-related (G3-P[3]-I3-R3-C3-M3-A3/A12-N3-T3-E3-H3/H6) genotype constellation [26, 27].

The segmented nature of the rotavirus genome provides a unique mechanism for the generation of genetic diversity via genetic reassortment. This occurs during mixed infections where the packaging of viral segments into sub-particles can lead to mixing genes from different viruses [28]. RV able to undergo genetic reassortment resulted in novel strain production by gene exchanged between human-animal strain or the interspecies transmission of animal strains to humans [20].

RV disease burden

RV infections were responsible for approximately 128,515 deaths annually among children younger than 5 years [3, 4]. Rotaviruses are very resistant to environmental conditions and highly contagious. The primary mode of transmission is the fecal-oral route. Transmission can also occur by ingestion of contaminated food, drinking contaminated water, and by touching contaminated hands or contaminated surface [29]. Rotavirus infection is seasonal, with a peak of incidence in winter/spring in temperate countries [30]. Clinical symptoms include acute diarrhea for 2–3 days,

fever, vomiting, malaise, anorexia and dehydration [31]. Death from RV disease is mainly due to severe dehydration and cardiovascular failure [32].

There are six most predominant strains of RVA species account for more than 90% of globally circulating RVA: G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] [33, 34]. G1P[8] is still the most predominant strain in the world followed by G2P[4] and G3P[8] [35]. There was an outbreak of RV infection both in children and adult in the winter season 2018 in Thailand. G9P[8] was the most predominant strain in adults, while in children G8P[8] was dominant [36, 37]. Global RV mortality rate has decreased by nearly 65% since 1990 because of improvements in clean water, sanitation, and nutritional status among children younger than 5 years old [38].

RV Seasonality

Meteorological conditions have an indirect yet important impact on the epidemiology of human rotavirus infection. Weather-related low indoor relative humidity and indoor crowding may be key factors in the epidemiology of rotavirus disease. Hospitalizations for rotavirus gastroenteritis tended to be more common after a cold or dry month than after a warm or wet corresponding calendar month [39].

The seasonal pattern in RV varies by climatic region and is also associated with local weather. A reduction in RV rates was associated with increased temperature and precipitation [30]. There is a significant association between increased numbers of estimated positive RV cases and lower humidity, rain, and temperature. In children younger than two years old, RV was the pathogen most frequently identified in the winter, dry, or cool/dry seasons [40]. In tropical climates, the

higher temperature was associated with a greater decrease in RV than in humid mid-latitude climates [41].

RV vaccine

The World Health Organization (WHO) recommended RV vaccines to be included in immunization programs in the European region and the Americas in 2006 then, in 2009, the WHO recommended the use of RV vaccines in all National Immunization Programs (NIPs). There are four globally available WHO-prequalified oral vaccines (Rotarix and RotaTeq, Rotavac and Rotasiil) at the end of 2018 [42]. Among all of RV vaccine, Rotarix and RotaTeq have been licensed in more than 100 countries. Rotarix is a two-dose monovalent vaccine consist of G1P[8] strain, while RotaTeq is a three-dose pentavalent vaccine consist of G1, G2, G3, G4 and P[8] RV strains [43]. As of November 2021, more than 110 countries introduced RV vaccine in the NIP with total global coverage reach 39% [44, 45]. In Thailand, national RV vaccine has been implemented since January 2020 [46].

RV vaccination does not completely protect young children against infection, but it reduce the severity of rotavirus-associated gastroenteritis (RVGE) [47]. RV vaccines are highly effective in preventing severe gastroenteritis in young children during the first 5 years of their life, particularly in developed countries [48]. The social economic status (SES) of a country seems to influence RV vaccine effectiveness [49]. Vaccination was predicted to prevent 93%, 86%, and 51% of severe RVGE in high, middle, and low SES, respectively [50]. Analysis of the data for the Asia region found median vaccine effectiveness of 94% in low child mortality countries, 64% in medium child

mortality countries, and 49% in high child mortality countries [43]. Factors that might contribute to this phenomenon include gut microbiota, genetic factors, transplacental antibodies, enteric pathogens, and environmental enteropathy [51, 52]. Evidence suggests that vaccine efficacy may vary by setting, due to regional differences in circulating RV vaccine strains and reduced efficacy of oral vaccines in settings with a high prevalence of malnutrition and gastrointestinal infections [53]. Pooled efficacy estimate of Rotarix and RotaTeq against severe RVGE in industrialized countries is 88% during the first year of age and 83% during the second year. However, RV vaccine efficacy is much lower in countries where the mortality rate for children under five years of age is high [54]. The efficacy of Rotarix and RotaTeq in the U.S. depends on the level of exposure during the RV season [55]. It can be concluded that vaccine efficacy is affected by individual factors such as nutritional level, gut microbiota, genetic factor, transplacental antibody and environmental enteropathy and external factors including SES, circulating vaccine strain, childhood mortality rate, and RV season in each country.

Additionally, RV vaccination confers herd protection among infants and children under 5 years old who had not been vaccinated [56, 57]. In developing countries with lower RV vaccine efficacy and coverage, indirect protection gain from herd immunity is more significant than in industrialized countries where vaccine efficacy and coverage exceed 90% [54]. It is predicted that vaccine introduction will result in an increase in selective pressure leading to changes in the strain distribution to escape immunity and will affecting the evolution of these strains [58]. Vaccine-driven strain replacement is a major concern after nationwide rotavirus vaccine introductions [59]. RV vaccine contain live attenuated virus. Possible horizontal transmission of a live attenuated vaccine

and the potential environmental spread of new reassortant strains leading to development of new infections [60]. Although it also carries a risk of causing infection in immunosuppressed patients, transmission of the attenuated vaccine strain from vaccinated to non-immunized children contributing greatly to the protection of a population by lead to herd immunity [61, 62].

New vaccine formulation and format were developed to overcome the limitation of RV vaccines. To avoid the risk factor of oral vaccine, parenteral administered RV vaccine are currently under development. Parenteral vaccine can be manufactured at a lower cost and are easier to transport as they are thermally stable, thus further reducing costs [63-65].

As a major pediatric enteric virus, the epidemiological study of RV infections is important to design the control measures. Documenting changes in rotavirus genotype prevalence, recognizing emergence of rare or unusual genotypes, and identifying potential vaccine escape strains will inform future vaccination strategies and the development of new rotavirus vaccines.

Significance of the study

TaqMan probe real time RT-PCR targeting the NSP3 gene is a specific method for RV screening. Multiplex RT-PCR provided robust, accurate, efficient, affordable, and documentable typing system. Combination of these methods will provide accurate information about RV genotype in an efficient time and value.

The diversity of RV genotypes may have significant implications for vaccine development and successful implementation, especially if strains that are not targeted by current vaccine candidates emerge as common types, either globally or regionally. To successfully develop and implement the rotavirus vaccines, an understanding of rotavirus epidemiology is needed in countries contemplating introducing vaccines. The predominant genotypes can vary unpredictably from year to year in any single location. The development of RV vaccines has prompted many countries to establish a program to assess the disease burden associated with RV infection and RV strain distribution. Strain surveillance helps to determine whether the most prevalent local strains are likely to be covered by the serotype antigens found in current vaccines.

In Thailand, where RV vaccine introduce in National Immunization Program (NIP) started in January 2020, surveillance program will be important to monitor the possibility of horizontal transmission, to determine if some strains escape immunity induced by the vaccines, whether rare strains emerge and if vaccine strains reassort or circulate in the population.

Hypothesis

1. Rotavirus is still the most common diarrhea virus causing AGE, with G3P[8] as the most predominant genotype.
2. Deep analysis by whole genome sequencing to monitor the emerging of unusual strain will find evidence of genetic reassortment and interspecies transmission.
3. Vaccine introduction will result shifting in predominant genotype and vaccine viral shedding can be found.

CHAPTER III

Rotavirus Infection in Children in Southeast Asia 2008-2018: Disease Burden, Genotype Distribution, Seasonality, and Vaccination

Publication

Lestari FB, Vongpunsawad S, Wanlapakorn N, Poovorawan Y. Rotavirus infection in children in Southeast Asia 2008-2018: disease burden, genotype distribution, seasonality, and vaccination. J Biomed Sci. 2020 May 21;27(1):66. doi: 10.1186/s12929-020-00649-8. PMID: 32438911; PMCID: PMC7239768.

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Abstract

Background: Rotaviruses (RVs) are recognized as a major cause of acute gastroenteritis (AGE) in infants and young children worldwide. Here we summarize the virology, disease burden, prevalence, distribution of genotypes and seasonality of RVs, and the current status of RV vaccination in Southeast Asia (Cambodia, Indonesia, Lao People's Democratic Republic, Malaysia, Myanmar, Philippines, Singapore, Thailand, and Vietnam) from 2008 to 2018.

Methods: Rotavirus infection in Children in Southeast Asia countries was assessed using data from Pubmed and Google Scholars. Most countries in Southeast Asia have not yet introduced national RV vaccination programs. We exclude Brunei Darussalam, and Timor Leste because there were no eligible studies identified during that time.

Results: According to the 2008–2018 RV surveillance data for Southeast Asia, 40.78% of all diarrheal disease in children were caused by RV infection, which is still a major cause of morbidity and mortality in children under 5 years old in Southeast Asia. Mortality was inversely related to socioeconomic status. The most predominant genotype distribution of RV changed from G1P[8] and G2P[4] into the rare and unusual genotypes G3P[8], G8P[8], and G9P[8]. Although the predominat strain has changed, but the seasonality of RV infection remains unchanged. One of the best strategies for decreasing the global burden of the disease is the development and implementation of effective vaccines.

Conclusions:

The most predominant genotype distribution of RV was changed time by time. Rotavirus vaccine is highly cost effective in Southeast Asia countries because the ratio between cost per disability-adjusted life years (DALY) averted and gross domestic product (GDP) per capita is less than one. These data are important for healthcare practitioners and officials to make appropriate policies and recommendations about RV vaccination.

Keywords:

Rotavirus, Disease burden, Genotypes, Vaccination, Southeast Asia

Introduction

Rotavirus (RV) History

RV was first identified in cattle in 1969 [8]. The virus appeared similar to those that cause diarrhea in mice [9], calves [10], and a virus identified from a rectal swab of a healthy monkey [11]. In May 1973, Bishop, Davidson, Holmes, and Ruck examined ultrathin sections of duodenal mucosa from children with acute gastroenteritis (AGE) by electron microscopy (EM), and found abundant viral particles in the epithelial cell linings of the upper villous surface which were similar in appearance to the RVs discovered in animals before [12]. EM also revealed 70-nm particles in negatively stained fecal extracts [13]. The viral particle was initially identified by several names including reovirus-like, orbivirus-like, duovirus, infantile gastroenteritis virus, or a "new" virus. The wheel-like structure observed on EM eventually led to the naming concensus of Rotavirus (*rota* is Latin for wheel) [14]. RVs have now been shown to be a cause of diarrhea in the young of many mammalian and avian species [66].

RV Morphology

RVs are 70-nm, non-enveloped RNA viruses belonging to the family *Reoviridae*. The RV genome consists of 11 segments of double-stranded RNA (dsRNA) surrounded by a triple-layered capsid. Each genomic fragment encodes protein of different function. The outer layer proteins (viral protein [VP] 4 and VP7) mediate attachment and penetration; the inner layer is composed of VP2 protein and encloses the viral genome and the minor protein VP1, the viral RNA-dependent RNA

polymerase, and VP3, the viral capping enzyme. The middle layer is composed of VP6 which interacts with and stabilized the inner and outer layer [16]. VP6 defines species/group and subgroup specificities [17-19]. All RNA segments, except for segment 11, are monocistronic, encoding either structural viral proteins (VP1 to VP4, VP6, and VP7) or non-structural proteins (NSP1 to NSP5). Genome segment 11 codes for two proteins: NSP5 and NSP6 [16]. RVs can be differentiated by a dual classification system, based on the two outer capsid proteins, VP7 and VP4, that determine the G (VP7, glycoprotein) and P (VP4, protease-sensitive) genotypes [20]. At least 36 G types and 51 P types have so far been identified in humans and animals [67]

A whole genome-based genotyping system was recently proposed for RV Group A (RVA) based on the genotype assignment of all 11 gene segments [25]. The genome of individual RV strains is given the complete descriptor of Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx to identify the genotypes of the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 encoding RNA segments, respectively. Most strains demonstrate either a Wa-like (G1-P[8]-I1-R1-C1-M1-A1-N1-T1- E1-H1), DS-1 (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2), or AU-1-related (G3-P[3]-I3-R3-C3-M3-A3/A12-N3-T3-E3-H3/H6) genotype constellation [26, 27].

Indirect immunofluorescence techniques targeting VP6 are used to differentiate RV species. RVs are currently differentiated into at least nine species, designated A to I and a tentative tenth species, J. RVA infects in birds and mammals; RVB, RVC, RVE, RVH, and RVI have been detected in one or more mammalian hosts; RVD, RVF, and RVG have been detected only in birds; RVJ infects bats [68-70]. **Table 1** shows rotavirus groups and its host.

Table 1. *Rotavirus groups and hosts.*

Rotavirus Group	Host
	Human, Pig [71], Cattle, Horse [72], Rabbit [73], Alpaca [74], Turkey, Pheasant, Bat, Sugar Glider, Camel, Vicugna, Velvet Scoter, Fox, Common Gull, Chicken, Shrew, Racoon, Mouse [8, 26, 75]
A	Sheep, Partridge, Panda, Monkey, Mussel, Oyster, Shellfish, Salmon, Shark, Trout, Deer, Mosquito, Cormorant, Fly, Moth, Tick, Tasmanian Devil, Leafhopper, Buffalo, Antelope, Dog, Civet, Cat [76] Giraffe [77] Pigeon, Guanaco, Macaques [78]
B	Human, Cattle, Pig, Rat, Goat [79]
C	Human [80], Dog, Bear, Ferret, Pig [81]
D	Chicken, Duck, Pigeon, Guinea Fowl [82]
E	Pig [83]
F	Pig, Chicken, Teal, Partridge [82]
G	Chicken, Duck, Pigeon, Turkey, Partridge, Gull, Avaret, Teal [82]
H	Human, Pig, Bat [84, 85]
I	Cat [86], Dog [69]
J	Bat [70]

RV Infection Burden

RVs were recognized as a major cause of AGE in infants and young children in 1973 [12, 87]. RV is the leading cause of diarrhea-associated mortality among children younger than 5 years, although the burden of RV has decreased during the past decade. RV infections were responsible for approximately 128,515 deaths annually among children younger than 5 years. RV constitutes 1 of the 13 diarrhea etiologic agents measured in the 2016 Global Burden of Disease Study [3]. It is the most prevalent agent causing severe diarrhea in both developed and developing countries [32, 88]. After RV vaccine introduction in developed countries, norovirus become the predominant viral pathogen that caused AGE in children. Norovirus prevalence remained stable or increased, whereas rotavirus activity dramatically decreased [89-91]. Nevertheles, from 2000 to 2013 in Southeast Asia, approximately 50.7% (n=10,765) of total diarrhea mortality was associated with RV disease [92].

Figure 2 shows the prevalence and death caused by diarrheal disease and RV in children under 5 years old from 1990 to 2017 in Southeast Asia. The prevalence of diarrheal diseases in Southeast Asia countries varies, but the mortality trend associated with diarrhea and especially RV infection has been decreasing in recent years. Lao People's Democratic Republic [PDR] reports one of the highest death rates in this period. However, improvement in hygiene and sanitation combined with the introduction of the rotavirus vaccine has contributed to decreasing RV infection [93].

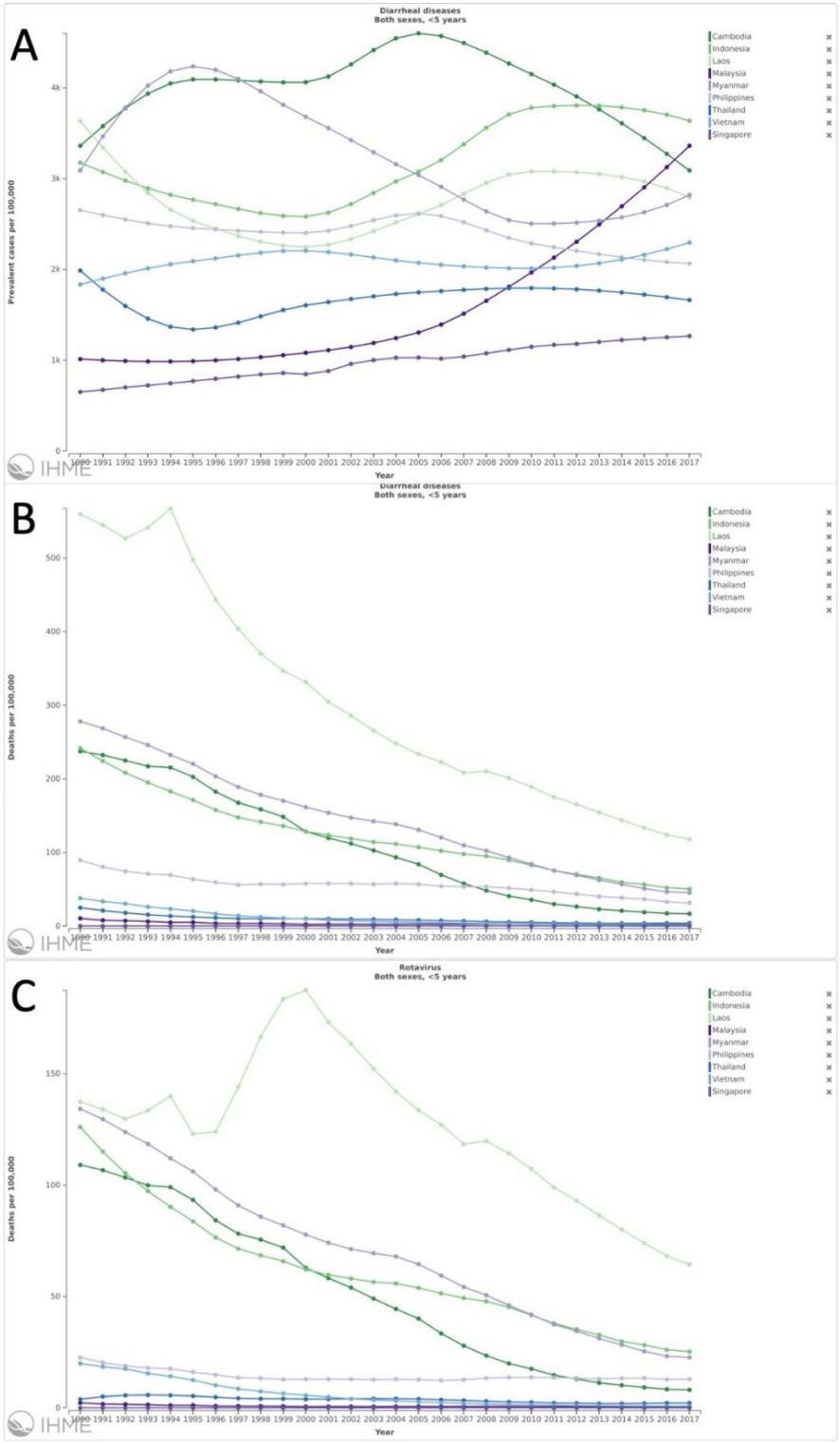

Figure 2. *Diarrhea diseases in children under 5 years old in Southeast Asian countries from 1990 to 2017.*

(A) Prevalence of diarrhea; (B) diarrhea-associated mortality; (C) mortality attributed specifically to rotavirus [38, 94].

We collected RV surveillance data from 9 countries in Southeast Asia (Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Singapore, Thailand, and Vietnam) for the years 2008–2018 to estimate the proportion of RV gastroenteritis (RVGE) **(Table 2)**. A total of 52,579 stool samples were collected of which 21,444 (40.78%) were RV positive. Acute diarrheal disease caused by RV is still a major cause of morbidity and mortality in children under 5 years old in developing countries, which may be attributed to the regions' lower standards of living and hygiene conditions [93]. However, our study revealed that as the gross domestic product (GDP) per capita increases, and the economic status of Southeast Asian countries improves, the RV mortality rate steadily declines (**Figure 3**). Higher socioeconomic status (SES) can improve sanitation, hygiene practices, and healthcare facilities to support better living conditions and decrease the RV mortality in children.

Table 2. *The annual incidence of rotavirus in children under 5 years old in Southeast Asian countries, 2007-2018*

Country	Region	Year	Study Design	Number of Stool Sample	Number of Rotavirus Positive	Percentage (%)	Reference
Cambodia	Phnom Penh	2010-2016	Active hospital surveillance	7007	3473	49.56	[95]
Indonesia	Bandung, Yogyakarta, Mataram, Denpasar	2009-2010	Hospital-based surveillance	4235	2220	52.42	[96]
	Yogyakarta	2009	Hospital-based surveillance	104	57	54.81	[97]
	Denpasar	2009-2011	Hospital-based surveillance	656	327	49.85	[98]
	Bandung	2009-2012	Prospective cross-sectional study	135	92	68.15	[99]
	Mataram	2010	Cross-sectional study	328	210	64.02	[100]
	Surabaya	2013	Cross-sectional study	220	88	40	[101]
	Pekanbaru	2015	Cross-sectional study	71	42	59.15	[102]
	Indonesia	2010-2015	National surveillance	4013	1950	48.59	[103]
	Surabaya	2015-2016	Hospital-based surveillance	134	42	31.34	[104]
	Central Java	2013-2016	Hospital-based surveillance	1649	105	6.37	[105]
	Jawa Timur	2015-2018	Hospital-based surveillance	432	137	31.71	[106]
Lao PDR	Vientiane	2009-2015	Hospital-based surveillance	1772	928	52.37	[107]
Malaysia	Malaysia	2008-2010	Hospital-based surveillance	822	279	33.94	[108]
Myanmar	Yangon	2009-2014	Prospective active surveillance	3724	1860	49.95	[109]

Philippines	Palawan	2012	Not specified	45	25	55.56	[110]
	Philippines	2013-2015	National Surveillance	5229	2024	38.1	[111]
	Zamboanga city	2016	Hospital-based surveillance	93	56	60.22	[112]
Singapore	Singapore	2008	Hospital-based surveilance	285	167	58.60	[113]
	Singapore	2008	Randomized clinical trial	58	11	18.97	[114]
Thailand	Bangkok, Khon Kaen, Nahon Ratchasima, Tak	2007-2009	Hospital-based surveillance	557	158	28.37	[115]
	Chiang Rai, Nakhon Ratchasima, Surat Thani, Phitsanulok,	2008-2010	Regional surveillance	3470	458	13.20	[116]
	Khon Kaen & Bangkok	2009-2011	Hospital-based surveillance	562	250	44.48	[117]
	Thailand	2010-2013	Active surveillance	1032	184	17.83	[118]
	Khon Kaen, Bangkok	2011-2014	Hospital-based surveillance	688	204	29.65	[119]
	Chiang Mai	2012	Hospital-based surveillance	186	35	18.82	[120]
	Nonthaburi	2012-2014	Hospital-based surveillance	No Data	73	-	[121]
	Sukhothai, Petchabun	2013-2014	Regional surveillance	2754	666	24.18	[122]
	Thailand	2014-2016	Hospital-based surveillance	1867	514	27.53	[123]
	Chiang Rai	2015-2016	Hospital-based surveillance	270	91	33.70	[124]
Vietnam	Ho Chi Min	2009-2010	Hospital-based surveillance	1419	664	46.79	[125]
	Vietnam	2012-2015	National Surveillance	8689	4054	46.66	[126]

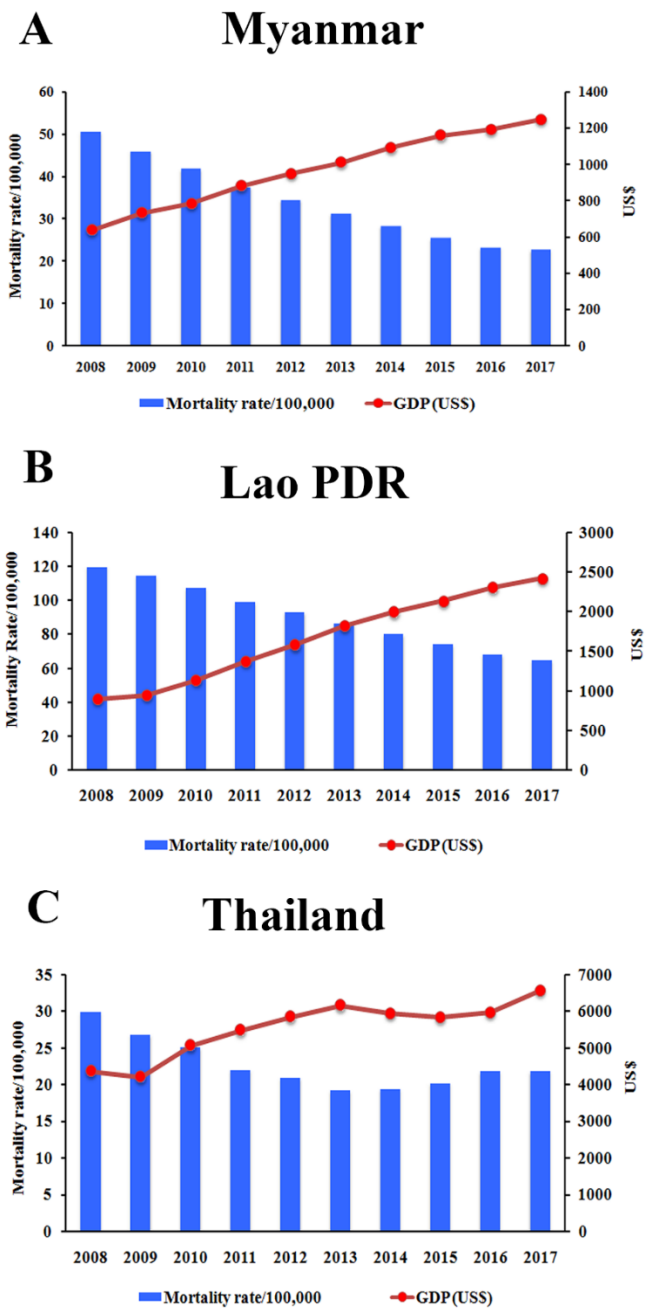

Figure 3. *The mortality of rotavirus-associated acute gastroenteritis per 100,000 children under 5 years old and the national gross domestic product (GDP) per capita between 2008 to 2017 in lower-middle income countries.*

(A) Myanmar and (B) Lao PDR, and in the upper-middle income country; (C) Thailand [94, 127]. The bar graphs represent the mortality rate per 100,000 populations. The red dots represent the GDP per capita in US$.

RV Genotype Distribution

Among the data from Southeast Asia countries examined, the most predominant genotype distribution of RV has changed except in Lao PDR and Malaysia. In 2009–2013, G1P [8] and G2P [4] were the most predominant genotypes, but starting 2014, it changed into the rare and unusual genotypes G3P[8], G8P[8], and G9P[8]. Several uncommon RV genotypes such as G2P[8], G8P[6], G5P[19], G9P[4], G9P[6], and G1P7[5] were identified in the surveillance data. The presence of such diversity among RV isolates provides insight into the evolution of these strains, which can arise due to point mutations, genetic rearrangements, reassortment events, and interspecies transmission [20, 33, 128]. Circulating RV strain appears diverse despite RV vaccination, which may enable the increase in the prevalence of non-vaccine strains. Thus, the circulation of strains in which vaccines have lower efficacy eventually impairs vaccine effectiveness [129]. In the Philippines, where the Rotarix® vaccine was introduced in July 2012, the frequency of RVGE cases caused by G1P[8] decreased while the circulation of G9P[8] increased significantly [127]. The genotype distribution of RV in Southeast Asia is shown in **Table 3.**

Table 3. *The distribution of rotavirus genotype in Southeast Asian countries based on the surveillance data from 2008 to 2018**

Genotype	Year									
	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017/ 2018
Cambodia										
G1P[8]			118	109	190	107	52	81	8	
G2P[4]				2	12	199	88			
G3P[8]			21	50	5	4		43	101	
G8P[8]				2	1	4	42	118	8	
G9P[8]			2			4	1	29	29	
others			19	3	34	52	18	17	14	
Indonesia										
G1P[4]		30	11	16		30				
G1P[6]		33	38	8		33				1
G1P[8]		64	219	98	138	202	2		1	12
G1P[UT]		3		1	3	6				
G2P[4]		35	25	17	1	36	1			
G2P[6]				5	7	7	7	4	5	
G2P[8]			4		3	3				
G3P[4]					1	1				
G3P[6]					1	1		1	4	19
G3P[8]		2			6	8	47	54	126	98
G3P[9]					1	1				
G3P[UT]									7	7
G9P[8]								1		

G12P[8]						8				
Thailand										
G1P[4]		8				4				
G1P[8]	14	217	63	146	87	236	447	42	3	
G2P[4]	38	107		3	5	109	152	3		
G2P[8]	1	11	2			4	1			
G3P[8]	1	22	20	156	26	4	19	125	44	
G3P[9]		1		2			3	2	1	
G4P[6]	1	1	2	1		1	1	1		
G8P[8]			1			58	164	8		
G9P[8]	7	58	7		13	1	5	77		
G9P[UT]		1	3				1			
G12P[6]	5	2	3		4					
G12P[8]		24	5							
Untypeable		5	1							
Myanmar										
G1P[6]		15	7	2	30		31	3		
G1P[8]		3			2					
G2P[4]		1	1	14	9	22	3	1		
G2P[6]						2	2			
G2P[8]								1		
G3P[8]		5								
G9P[4]					1	1	2			
G9P[8]					7	1	20	20		
G12P[6]		6	50	103	45	1				

G12P[8]		3	7	8	37	2				
Mixed		6	5	2	11			1		
Partially typed		4	4	7	26	1	14	10		
Untypeable			7	1	5		2	3		
Lao PDR										
G1P[4]		6					1	1		
G1P[8]		53	32	47	15	96	14	145		
G2P[4]		66	44	2	6	57	76	3		
G2P[8]		2	1			1				
G3P[4]			2							
G3P[8]		7	32	80	92	7				
G3P[9]				1						
G4P[4]		1								
G4P[6]				1						
G8P[8]								1		
G9P[4]			1							
G9P[8]			40			6		1		
G10P[4]							1			
G12P[6]					1					
Mixed		19	2			1	2			
Untypeable		11	1							
Philippines										
G1P[8]						232	543	417	587	
G2P[4]						55	101	400	187	
G9P[8]						19	51	43	360	

Untypeable						8	22	7	133	
Mixed						3	7	28	13	
Unusual						5		28	40	
G1P[6], G2P[6], G1P[9]								9	13	
Vietnam										
G1P[4]					12	12	9			
G1P[8]					934	985	391	125		
G2P[4]					58	108	306	234		
G3P[8]					47	24	68	50		
G8P[8]							9	259		
Mixed					23	12	34	58		
Untypeable					93	48	26	92		
Others						12	9	17		
Malaysia										
	2008-2010									
G1P[8]	206									
G2P[4]	19									
G9P[8]	16									
G12P[8], G3P[9], G9P[9], G3P[8]	10									
Singapore										
	2005-2008									

G1P[4]	4									
G1P[8]	125									
G1P[11	1									
G2P[4]	49									
G2P[8]	5									
G3P[4]	1									
G3P[8]	61									
G9P[4]	5									
G4P[8]	1									
G9P[8]	68									

*** In Singapore, the available data was between 2005 to 2008**

Differences in the predominance of RV genotypes and newly emerging strains were identified over the surveillance period in Cambodia. The G1P[8] genotype was predominant in 2010, 2011, and 2012 (74%, 66%, and 79%, respectively), whereas genotype G2P[4] predominated between 2013 (54%) and 2014 (44%). The previously uncommon strain G8P[8] also emerged in 2014 (21%). The proportion of G8P[8] genotype detections increased further in 2015 (41%), in conjunction with the emergence of G9P[8] (10%). By 2016, the detection of genotype G9P[8] had increased to 18%, and G3P[8] became the most prevalent genotype (responsible for 63% of detections) [95].

In Indonesia, the surveillance results demonstrated a changing trend for the most prevalent genotype, from G1P[8] in 2009-2013 to G3P[8] in 2014-2018. From 2009 to 2013, G1P[8] was the most prevalent genotype circulating, which accounted for 38%, 73.7%, 67.5%, 85.7%, and 60.1%

each year, respectively. G3P[8] became the most predominant strain in 2013, and this continued to 2015, accounting for 49,7%, 82.5%, and 84,4%, respectively [97, 101, 103-106]. Another study reported that G3P[8]/[6] was also the predominant strain during 2015-2018 [106].

During the 7-year surveillance period in Lao PDR, the most predominant genotypes identified by year were G2P[4] (40%) and G1P[8] (32%) in 2009; G2P[4] (28%) and G9P[8] (26%) in 2010; G3P[8] (61%) and G1P[8] (36%) in 2011; G3P[8] (81%) in 2012; G1P[8] (57%) and G2P[4] (34%) in 2013; G2P[4] (81%) in 2014; and G1P[8] (96%) in 2015 [107].

From 2008 to 2010, the most common genotype in Malaysia was G1P[8] (82%). Other genotypes identified were G2P[4] (7.6%) and G9P[8] (6.3%). Approximately 4% of the samples were either mixed or untypeable (G12P[8], G3P[9], G9P[9], G3P[8]) [108]. A 2006 preliminary report in Sabah State showed that approximately 33% of samples were positive for RV, of which 33% were of genotype G4P[8][130].

In Myanmar, the most common strains in 2009 were G1P [8] (28.3%) and G12P [8] (28.3%). G12P [8] was detected from 2009 to 2012, ranging from 28.3% in 2009 to 70% in 2011. G2P[4] became the most predominant strain in 2012–2013, followed by G1P[8] in 2013-2014. G9P[8] comprised only 1% of the RV strains in 2011 and increased to 97.5% in 2014. While in 2015, the majority (90%) of RV strains comprised G9P[8] (54%) and G3P[8] (36%). G9P[8] emerged in Myanmar in 2011 and was the most common strain in 2014 and 2015 [109, 131].

In the Philippines, 1949 (98.5%) RVA-positive stool specimens were successfully typed. The most common genotypes identified were G1P[8] (60.3%), G2P[4] (28.1%), and G9P[8] (5.7%). The

frequencies of RVGE cases due to G1P[8] were similar in 2013 (72.0%) and 2014 (75.1%), but it decreased to 44.7% in 2015. Likewise, the frequencies of cases due to G2P[4] were similar in 2013 (17.1%) and 2014 (14.1%) but increased to 42.9% in 2015. The proportion of RVGE cases caused by G9P[8] did not change appreciably from 2013 (5.9%) to 2014 (6.9%) or 2015 (4.6%). Mixed genotypes, unusual strains, and animal strains were detected in specimens from 38 (1.9%), 22 (1.1%), and 10 (0.5%) children with RVGE,. Rare and unusual genotype combinations identified include three G1P[4], eight G2P[8], seven G8P[6], two G8P[8], and two G9P[4] strains. Also, animal strains were detected in specimens from 10 children, including 1 G3P[9] feline-like and nine G4P[6] porcine-like strains [111].

The predominant strain observed in Singapore was G1P[8] (18,3%), while G9P[8] (9,9%) was the second most common type observed among children in Singapore [113, 114].

In Thailand, G2P[4] was the most common genotype in 2008 (53,5%). G1P[8] was the predominant genotype in 2009, 2010, 2012, 2013, and 2014, accounting for 47.3%, 35.8%, and 63.9%, 60.4%, and 56.2%, respectively. In 2011 (68.7%), 2015 (47.3%), and 2016 (89.8%), most RV strains were G3P[8]. Uncommon genotypes found were G1P7[5], G5P[19], G9P[4], and G9P[6], adding to the existing list of uncommon genotypes reported to circulate in Thailand [115-119, 121-124].

Based on RV diarrhea data from four sentinel hospitals in Vietnam from 2012 to 2015, G1P[8] was the most prevalent strain during 2012 and 2013, accounting for 80% and 82% of total genotyped samples, respectively. G2P[4] was found in 5% of samples in 2012 and 9% in 2013. In 2014 and 2015, the proportion of RVGE caused by G2P[4] increased to 36% and 28%, respectively.

G8P[8] was not detected in 2012 and 2013, it accounted for only 1% of specimens in 2014, and it became predominant (31%) in 2015 [126].

RV Seasonality

Meteorological conditions have an indirect yet important impact on the epidemiology of human rotavirus infection. Weather-related low indoor relative humidity and indoor crowding may be key factors in the epidemiology of rotavirus disease. Hospitalizations for rotavirus gastroenteritis tended to be more common after a cold or dry month than after a warm or wet corresponding calendar month [39].

The temporal trend of Cambodian RV infection shows substantial year-round transmission with prominent peaks during colder, dry months. Peaks typically occurred between November and May [95]. In Indonesia, RV infection was present throughout the year and did not demonstrate clear annual seasonality [103]. Conversely, infection generally peaks during the rainy season in Singapore and Malaysia. An outbreak of RV infection was observed from January to March [132]. Positive RV cases increased in number and proportion during the dry season (January–April) each year in Lao PDR [133]. In Myanmar and the Philippines, RV infection has a strong seasonal peak in colder, drier months, as seen in other Asian countries. The highest rate of RV infection occurred in January and February [109, 111]. RV cases in Thailand were most prevalent during the cooler months, specifically from January to March, but RV was detected every month in the northern part of Thailand, where the weather is relatively cooler compared to the rest of the country [116]. In

Vietnam, RV was detected every month, but most RV gastroenteritis (GE) cases occurred between December and May [126]. **Figure 4** shows the RV seasonality in Southeast Asian countries.

The seasonal pattern in RV varies by climatic region and is also associated with local weather. A reduction in RV rates was associated with increased temperature and precipitation [30]. There is a significant association between increased numbers of estimated positive RV cases and lower humidity, rain, and temperature. In children younger than two years old, RV was the pathogen most frequently identified in the winter, dry, or cool/dry seasons [40]. In tropical climates, the higher temperature was associated with a greater decrease in RV than in humid mid-latitude climates [41]. It had been suggested that the seasonal pattern may be driven by airborne transmission of the disease [134].

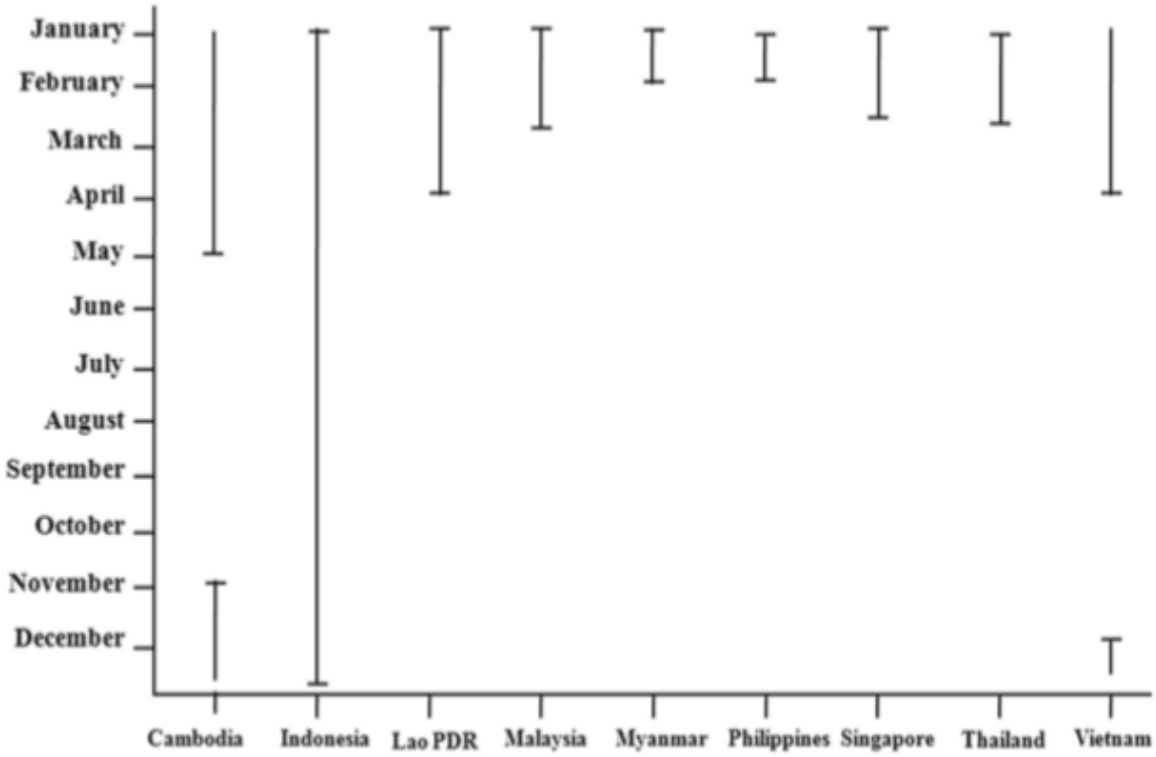

Figure 4. Seasonality of rotavirus in Southeast Asian countries.

RV Vaccination

One of the best strategies for decreasing the global burden of disease is the development and implementation of effective vaccines [32]. RV is the most common cause of vaccine-preventable severe diarrhea [5]. The World Health Organization (WHO) recommended that RV vaccines be included in immunization programs in the European region and the Americas in 2006. In 2009, following efficacy studies in low-income countries (LICs) and lower-middle-income countries (LMICs) in Africa and Asia, the WHO recommended the use of RV vaccines in all National Immunization Programs (NIPs) [135]. RV vaccines had been introduced in 101 countries by the end of 2018, and global coverage was estimated to be at 35% [136]. Most countries in Southeast Asia

have not yet introduced national RV vaccination programs. Among Asian countries, only the Philippines, and recently Thailand have introduced the vaccines on a limited basis [137].

The Global Alliance for Vaccines and Immunizations (GAVI), also known as the Vaccine Alliance, actively supports RV vaccination by subsidizing the cost in eligible countries. In LMIC, the budget impact is an important criterion for funding new interventions, particularly for large public health investments such as new vaccines. By the end of 2018, GAVI had funded RV vaccine introductions in 45 countries [138].

RV Vaccine History

Research to develop a safe, effective RV vaccine began in the mid-1970s when investigators demonstrated that previous infection with animal RV strains protected laboratory animals from experimental infection with human RVs [139]. The first multivalent live oral reassortant vaccine developed was RotaShield® (a rhesus RV tetravalent [RRV-TV] vaccine) in the late 1980s [140]. The RRV-TV vaccine was licensed in August 1998 for routine use in children in the United States at 2, 4, and 6 months of age due to its proven efficacy [141]. In 1999, RotaShield® was voluntarily withdrawn from the U.S. market due to an increased risk of intussusception within 3 to 14 days after the first dose in infants <3 months of age [142].

Due to the past association of intussusception and the earlier RV vaccine, large safety studies were performed both for RV1 and RV5 before market authorization [143]. Rotarix® vaccine were highly efficacious in protecting infants against severe rotavirus gastroenteritis [114,

144] and were not associated with an increased risk of intussusception [145-147]. The pentavalent rotavirus vaccine (RotaTeq®) was highly efficacious against severe rotavirus gastroenteritis and provided substantial protection against rotavirus gastroenteritis of any severity. A significantly increased risk of intussusception in vaccine recipients was not detected [148]. In February 2014, WHO reviewed global intussusception data and found that the risk of intussusception following current rotavirus vaccines remains small compared to the benefits of preventing the impact of severe diarrhea [149].

At the end of 2018 there are four globally available WHO-prequalified oral vaccines (Rotarix® and RotaTeq®, Rotavac® and Rotasiil®) [42], one rotavirus vaccine licensed in China (Lanzhou lamb RV vaccine), one in Vietnam (Rotavin-M1), and there are several candidates in development [150].

Two RV vaccines, Rotarix® and RotaTeq®, have been developed by Glaxo Smith Kline and Merck, respectively. Rotarix® is a live attenuated monovalent vaccine derived from the most common human RV strain, G1P[8]. RotaTeq® is a live attenuated pentavalent vaccine containing mono-reassortant strains with genes encoding the human G1, G2, G3, G4, and P[8] protein in the genetic background of a bovine RV strains [88, 151]. These vaccines are highly effective for the global prevention of severe diarrhea and are included in the NIPs or phased subnational introductions in 101 countries by the end of 2018 [152, 153].

Rotavac® (Bharat Biotech International Limited) is a monovalent human-bovine RV vaccine. The vaccine consists of the 116E RV strain, which is a naturally occurring reassortant strain G9P[11],

containing 1 bovine RV gene P[11] and 10 human RV genes [154]. Rotavac® is the first to be introduced into a public vaccination program as of April 2016 when it was introduced in four states in India [155]. Rotasiil® is a live attenuated human-bovine reassortant pentavalent RV vaccine that contains genes encoding the VP7 of serotypes G1, G2, G3, G4, and G9. In March and September 2018 Rotavac® and Rotasiil®, respectively achieved WHO prequalification. Rotavac® has a vaccine efficacy of 53.6% for severe RV diarrhea in India [154], while Rotasiil® has efficacies of 60.5% to 66.7% in India [156] and Niger respectively [157]. Rotasiil® can safely be delivered with decreased dependence on the availability of a cold chain [158].

The Lanzhou Institute of Biological Products manufactures the Lanzhou Lamb RV vaccine (LLR). It is a monovalent lamb vaccine strain G10P[12], attenuated by cell passage [159], and was licensed in China in 2000. When given to children between 9 and 35 months old, one dose of the LLR vaccine conferred partial protection [160]. Vaccine effectiveness in children under 5 years of age was recently estimated at 35% (13 to 52%) against RV diarrhea and 53% (15 to 75%) against moderate-to-severe RV diarrhea based on a large case-controlled study [161].

Rotavin-M1 vaccine is manufactured by the Center for Research and Production of Vaccines and Biologicals and was licensed for use in Vietnam in 2012. The vaccine was derived from an attenuated strain, G1P[8], isolated from a Vietnamese child. A clinical trial found the vaccine to be safe and immunogenic in Vietnamese infants [162].

Another candidate RV vaccine, RV3-BB, was developed from a neonatal strain G3P[6] identified in Australia, with ongoing early clinical studies conducted in New Zealand and now

underway in Indonesia. It was also successfully implemented for vaccination of neonates [163]. **Table 4** describes the comparison of all the RV vaccines developed and used so far.

Intussusception, neutralizing antibodies present in breast milk, as well as the lower vaccine effectiveness in less developed settings has stimulated interest in an alternative, parenteral approach to immunization [50, 164-166]. The inactivated rotavirus particles, protein sub-units or virus-like particles (VLPs, structurally-similar to live virus) are being investigated as rotavirus vaccine candidates [166-168].

Three types of animal models have been used to evaluate protective efficacy of VLPs: infection models (adult mice and rabbits), disease models (gnotobiotic piglets), and models evaluating passive protection (neonatal mice and calves) [169]. Gnotobiotic pig was used to assessed the immunogenicity and protection of a candidate inactivated rotavirus vaccine (IRV), the human strain CDC-9 (G1P[8] [170] and attenuated Wa human rotavirus {AttHRV] or non replicating Wa 2/6 rotavirus-like particles [171]. Mice, rabbits, and piglets were used to evaluate the efficacy of VPL such as 2/6-VLPs (consisting of VP2 and VP6) [169] and RF 8-2/6/7-VLPs [172]. Human clinical trial recently assessed in South African toddlers and infants was done for the subunit vaccine P2-VP8-P[8] [64, 173].

Table 4. **Comparison of rotavirus vaccines**

Rotavirus Vaccines	Rotarix (GSK) [151]	RotaTeq (Merck) [151]	Rotavac (Bharat Biotech) [154]	RotaSIIL (Serum) [158]	Rotavin (Polyvac) [162]	LLR (Lanzhou) [160, 161]	RV3-BB (Bio Farma) [163]
Licensure	Several countries, 2006	Several countries, 2006	India, 2014	India, 2017	Vietnam, 2012	China, 2000	Clinical trial phase IIb
Prequalification	Yes	Yes	Yes	Yes	No	No	No
Strains	Monovalent, human derived G1P[8]	Pentavalent, WC3 G6P[5] bovine reassortant G1-4,P8	Monovalent, human neonatal derived G9P[11]	Pentavalent, UK Bovine G6P[5], reassortant G1-4, G9	Monovalent, human G1P[8]	Monovalent, Lamb G10P[12]	Monovalent, human neonatal G3P[6]
Number of doses	2	3	3	3	2	3	3
Age, first dose	6 weeks	6 weeks	6 weeks	6 weeks	6 weeks	2 months	New born: 0-5 days Infant: 8 weeks
Age, last dose	24 weeks	32 weeks	14 weeks	14 weeks	14 weeks	36 months	New born: 14 weeks Infant: 18 weeks
Dosage	10^6 median $CCID_{50}$ of live attenuated human G1P [8] RV	2.0–2.8 × 10^6 infectious units per reassortant	10^5 fluorescent focus unit (FFU) of live rotavirus	$10^{5.6}$ infectious units per reassortant	$10^{6.3}$ FFU/dose of live attenuated human G1P[8] particles	>5.5 log $CCID_{50}$	8.3-8.7 × 10^6 FCFU/ml
UNICEF price per course for GAVI-supported countries, 2020 [173]	$4.58	$9.60 (RotaTeq is no longer an option available to GAVI-supported countries)	$2.55	$4.65 (1-dose vial) $2.85 (2-dose vial)	$17.60	$72 for the three-dose series	

Vaccine Efficacy

RV vaccination does not completely protect young children against infection, but it reduce the severity of RVGE [47]. RV vaccines are highly effective in preventing severe gastroenteritis in young children during the first 5 years of their life, particularly in developed countries [48]. The SES of a country seems to influence RV vaccine effectiveness [49]. Vaccination was predicted to prevent 93%, 86%, and 51% of severe RVGE in high, middle, and low SES, respectively [50]. Analysis of the data for the Asia region found median vaccine effectiveness of 94% in low child mortality countries, 64% in medium child mortality countries, and 49% in high child mortality countries [43]. Factors that might contribute to this phenomenon including gut microbiota, genetic factors, transplacental antibodies, enteric pathogens, and environmental enteropathy [51, 52]. Evidence suggests that vaccine efficacy may vary by setting, due to regional differences in circulating RV vaccine strains and reduced efficacy of oral vaccines in settings with a high prevalence of malnutrition and gastrointestinal infections [53]. Pooled efficacy estimate of Rotarix® and RotaTeq® against severe RVGE in industrialized countries is 88% during the first year of age and 83% during the second year. However, RV vaccine efficacy is much lower in countries where the mortality rate for children under five years of age is high [54]. The efficacy of Rotarix® and RotaTeq® in the U.S. depends on the level of exposure during the RV season [55]. It can be concluded that vaccine efficacy is affected by individual factors such as nutritional level, gut microbiota, genetic factor, transplacental antibody and environmental enteropathy and external factors including SES, circulating vaccine strain, childhood mortality rate, and RV season in each country.

Additionally, RV vaccination confers herd protection among infants and children under 5 years old who had not been vaccinated [56, 57]. In developing countries with lower RV vaccine efficacy and coverage, indirect protection gain from herd immunity is more significant than in industrialized countries where vaccine efficacy and coverage exceed 90% [54].

Vaccine Introduction in Southeast Asia

Many countries in Southeast Asia have not implemented national RV vaccination programs including Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Singapore, Thailand and Vietnam. One reason is because of uncertainties regarding the cost-effectiveness of incorporating RV vaccination into the NIP. In addition, the vaccine's decreased efficacy in LIC settings has discouraged its introduction. Prevention of diarrhea in these countries has focused on patient treatment and the management of water quality, sanitation, and hygiene [130].

In Indonesia, the RV vaccine has been commercially available since 2011. Indonesia's national vaccine manufacturer, PT. Bio Farma, Bandung, is developing an RV vaccine using G3P[6] strain in collaboration with the Murdoch Children's Research Institute in Melbourne, Australia [174]. Bio Farma is currently driving clinical development, intending to introduce the vaccine into the Indonesian NIP by 2021, and eventually develop a product for the global market [168].

Currently Rotarix® and RotaTeq® are commercially available in Malaysia through private health providers [175]. However, RV vaccine is not included in Malaysia NIP because it is not considered permissible under Islamic shariah law (halal) [130]. The current oral rotavirus vaccines

use porcine trypsin in the manufacturing process [176]. There are also concerns about competing public health priorities and price [130]. The Health Ministry recently said the RV vaccine would be included in NIP if the associated mortality rate for children aged 5 and below exceeded 10%. However, the childhood mortality rates in Malaysia was 0.5% in 2014 and 2.9% in 2015 [177].

In Myanmar and Lao PDR where individual incomes are relatively low, international assistance will support for the RV vaccine introduction in 2020. The total amount of GAVI support for Myanmar is $4,088,000 [178]. Lao PDR is also planning RV vaccine introductions into the NIP in near future and assistance will be provided the Asian Development Bank and GAVI through an accelerated transition program [130].

In July 2012, the Philippines became the first Asian country to introduce RV vaccines into its NIP. The Philippines has initially focus on immunizing children living in the poorest communities, which have the highest child morbidity and mortality rates from the diarrheal disease [179]. The target population was identified by the Department of Social Welfare and Development, but there were challenges with nationwide vaccine distribution. In 2014, vaccine introduction was limited to the Caraga region, where it was co-administered with oral polio vaccine and the pentavalent vaccine. By 2015, vaccine coverage was close to 90% in the province of Agusan del Sur within this region but subsequently decreased due to a supply shortage [130]. In Agusan del Sur, the RV vaccine became available to the poorest quintile in September 2012; in January 2013, availability was expanded to all age-eligible children in two municipalities, San Francisco and Prosperidad; it was available to the entire province in July 2014. RV vaccine introduction was associated with a

substantial decline in diarrheal hospitalizations and outpatient consultations for diarrhea in Agusan del Sur, Philippines [180].

Two live-attenuated, orally administrable RV vaccines Rotarix® and RotaTeq® were licensed in Singapore in October 2005 and July 2007, respectively [147]. To date, RV vaccination is optional in Singapore [113].

The National Vaccine Committee of Thailand considered the introduction of an RV vaccine in 2010 in Sukhothai and Petchabun. Sukhothai province began a routine immunization program with an RV vaccine in October 2011. Evaluation of the first introduction was completed in 2017 and concluded that RV vaccine was highly effective in preventing diarrheal hospitalizations and conferring herd protection among older children who had not been vaccinated [56].

Vietnam is located in a region of high RV infection incidence and eligible for financial support to introduce vaccines into the expanded program of immunization (EPI) from the GAVI [181]. In 2012, the local vaccine manufacturer Polyvac licensed Rotavin-M1, which is based on an attenuated G1P[8] strain. Rotavin-M1 will be offered to children less than 1 year through a two-dose schedule, vaccinating infants at 2 and 4 months [162]. Rotarix® and RotaTeq® are also licensed in Vietnam and are available in the private sector, with approximately 590,000 doses imported since 2017. That same year, the Government approved the introduction of RV vaccination into Vietnam's NIP by September 2019 with GAVI support. In 2021, the national government will pay for 80% of the vaccine cost, while GAVI will cover the remaining 20% and all operational costs. By 2022, all costs will be covered by the government [130].

Health and Economic Impact of RV Vaccination

In LMICs, understanding the short- and long-term impact of intervention adoption on national budgets is critical for ensuring program sustainability [182]. Both budget impact and cost effectiveness are key criteria, among others, for policy makers deciding how to allocate limited resources [183]. Vaccination would be considered a worthwhile investment for improving general childhood development and health levels in most LIC. The highest reduction in burden would be achieved in countries with a high disease burden (≥200 RV deaths per 100,000 children under 5 years old), but a similar reduction would be achieved in countries with a medium burden (100–200 RV deaths per 100,000 children under 5 years old) because disease burden reduction also depends heavily on population size and country-specific vaccine efficacy adjusted for local RV serotype distributions [184].

For GAVI non-eligible countries, the price for Rotarix® is $2.49 – 7.27, and for RotaTeq® is $3.65 – 5.09 [185]. For GAVI-eligible countries, the price per dose will depend on the country's gross national income per capita averaged over the previous 3 years. As such Indonesia, Malaysia, the Philippines, Singapore, and Thailand are fully not eligible for GAVI vaccine prices and will have to rely on self-financing [186]. For LIC in Asia, introducing vaccines would halve RV-related deaths and medical visits, leading to significant cost reductions [187].

WHO-CHOICE (CHOosing Interventions that are Cost-Effective) uses the GDP as an indicator to develop the following widely referenced categories of cost-effectiveness. Disability-adjusted life

years (DALYs) averted is a widely used indicator that allows easy comparison with a 'no vaccination' strategy and with others public health interventions. The ratio between costs per DALY averted and GDP per capita less than one is defined as highly cost effective. The ratio between 1 to <3 and ≥3 are defined as cost effective and not cost effective, respectively [188]. The lowest and highest GDP values were in Myanmar with $1326 and Malaysia with $11,239, respectively [189]. **Figure 5** shows the comparison between costs per DALY averted with each country's GDP in 2018. We excluded the Philippines because the RV vaccine already included in the country's NIP and Singapore because Singapore is a high-income country. According to categories of cost-effectiveness, RV vaccine introduction into Southeast Asia countries is highly cost-effective because the ratio between costs per DALY averted and GDP per capita it is less than one.

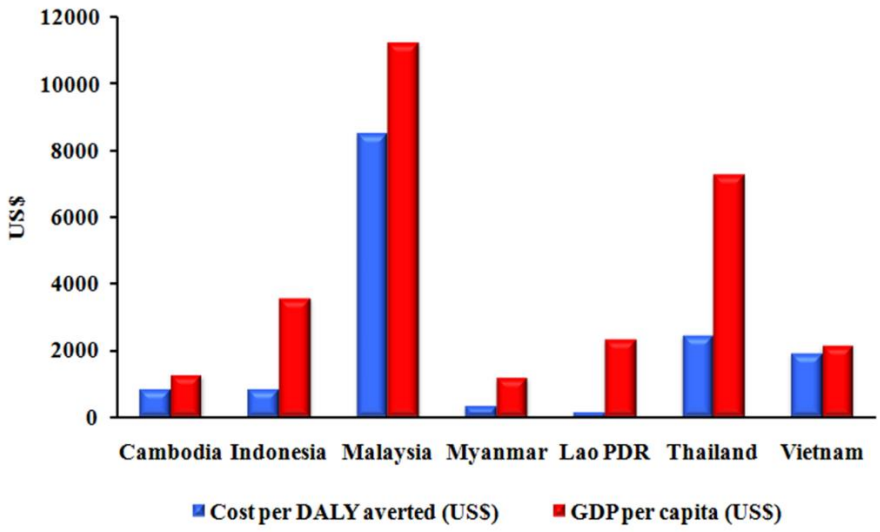

Figure 5. *Comparison between cost per DALY averted and GDP per capita in Southeast Asia. Introducing the rotavirus vaccine in Southeast Asia is highly cost-effective because the ratio is less then one [49, 148, 189, 190].*

Conclusions

According to 2008–2018 RV surveillance data for Southeast Asia, 40.78% of all diarrheal cases were causes by RV. Acute diarrheal disease caused by RV is still a major cause of morbidity and mortality in children under 5 years old in Southeast Asia. Among all assessed countries, the most predominant genotype distribution of RV changed from G1P[8] and G2P[4] into the rare and unusual genotypes G3P[8], G8P[8], and G9P[8]. Although the predominant RV strain has been changed, but the seasonality of RV infection remains unchanged. Continuous surveillance is necessary to determine whether they are regional genotype differences. Epidemiological data on RV prevalence will greatly facilitate vaccine development. In the mean time, the development of new vaccines will be needed if RVs are able to evade current vaccine immunity. More effective vaccines may also further decrease RV infection in children in LIC and LMIC, where currently available vaccines provide moderate efficacy. RV vaccine efficacy is affected by individual factors and external factors. Although most countries in Southeast Asia have not yet introduced national RV vaccination programs, such introduction is projected to be highly cost-effective because the ratio between costs per DALY averted and GDP per capita it is less than one.

CHAPTER IV

Childhood diarrhea associated with recent RotaTeq rotavirus vaccination in Thailand

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Abstract

Rotavirus (RV) infection remains one of the major causes of viral diarrhea in young children worldwide. Despite the success of RV vaccines including RotaTeq in significantly reducing morbidity and disease severity associated with hospitalization in developed countries, national immunization against RV have only just begun in Thailand in 2020. Consequently, possible RV vaccine shedding among pediatric vaccine recipients has not been rigorously documented here. During the coronavirus pandemic of 2020 and 2021, we received 257 diarrhea samples from four sentinel hospitals in Thailand. Only 25 samples (9.7%) tested positive for RV and G3P[8] was the predominant genotype. Six samples contained multiple RV strains based on detailed sequence analysis of the VP7 and VP4 genes, of which two samples possessed RV with genetic similarity to the vaccine strains in RotaTeq. Genome constellation of one sample (B8019) was consistent with G1P[8] vaccine strain reassortant. Another sample (B7711) contained G1, G2, G3, G4, P[5], and P[8] vaccine strains, as well as equine-G3P[4] wild-type RV. To our knowledge, neither report of diarrhea from RV infection after RotaTeq vaccination nor simultaneous shedding of vaccine-derived and wild-type RV infection has previously been described in Thailand. These results suggest the need for increased awareness of RV-associated diarrhea following routine vaccination and demonstrate evidence of possible co-infection with wild-type RV shortly after vaccination.

Introduction

Rotavirus (RV) infection causes approximately 128,000 deaths among children younger than 5 years of age worldwide [38]. RV belongs to the *Reoviridae* family and has 11 segmented double-stranded RNA genomes encoding the structural (VP1 to VP4, VP6 and VP7) and non-structural (NSP1-NSP5) genes [66]. Epidemiological surveillance using molecular methods involves characterizing at least two structural outer capsid protein genes, VP7 and VP4. Genotyping of VP7 (G, glycoprotein) and VP4 (P, protease-sensitive) often precedes the determination of all gene segments in the order VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 into the corresponding genome constellation Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, respectively [26]. To date, there are 41 G, 57 P, 31 I, 27 R, 23 C, 23 M, 38 A, 27 N, 27 T, 31 E, and 27 H genotypes among RV identified in human and animal species worldwide [23].

Vaccination against RV can significantly decrease the burden of morbidity and mortality associated with hospitalization [191]. There are two globally approved live-attenuated oral RV vaccines, which many countries have incorporated into their immunization programs. Rotarix is a monovalent vaccine derived from the most common human RV strain G1P[8] [192]. RotaTeq is a pentavalent vaccine containing five human-bovine reassortant strains (G1, G2, G3, G4, and P[8]) on the backbone of the naturally attenuated tissue culture-adapted parental bovine rotavirus (BRV) strain WC3 [193].

While Rotarix requires two doses and are recommended at 2 and 4 months of age, RotaTeq is administered on a 3-dose schedule at 2, 4, and 6 months of age [194]. Since both Rotarix and

RotaTeq are live vaccines, they can replicate and are shed in the feces after vaccination [195]. Prior studies have shown that post-vaccination shedding of RV vaccine strains in stools are detected in up to 50% and 10% of the Rotarix and RotaTeq vaccine recipients, respectively [61, 196]. The duration of viral shedding after the first dose of RotaTeq ranges between 1 to 15 days in 9% of vaccinated healthy children [197]. Studies from Malawi and Taiwan have reported viral shedding of up to 4 weeks post-vaccination [198, 199]. Immunocompromised patients can experience prolonged viral shedding up to one year after the last dose of RotaTeq [200]. Not surprisingly, vaccine-derived RV transmission associated with viral shedding from vaccinated to unvaccinated children have been reported [201].

RV vaccination among Thai children was uncommon prior to February 2020 when it was included in the national immunization program [46]. Thus, the annual incidence of RV infection resulting in hospitalization could have been lower prior to 2020 had RV vaccine use been more widespread. Coincidentally, the global coronavirus pandemic beginning in 2020 had inadvertently reduced the incidence of RV as children were kept away from schools and social distancing blunted viral transmission [202]. Nevertheless, intermittent easing of social restrictions after periodic declines in coronavirus infections (including students returning to schools) has resulted in the resurgence of RV infection. As part of the RV surveillance program, we undertook a study to determine the prevalence of RV infection during the coronavirus pandemic in the 2020-2021 calendar year. We subsequently identified RV infections associated with recent RotaTeq vaccination in pediatric vaccine recipients in Thailand, which up till now has not been well-documented.

Materials and Methods

Samples

A total of 257 stool specimens were submitted during January 2020 and December 2021 from four hospitals serving as sentinel sites for this study. They were King Chulalongkorn Memorial Hospital (eastern Bangkok), Bangpakok 9 International Hospital (western Bangkok), Bangkok Hospital Pitsanulok (Pitsanulok province), and Chumphae Hospital (KhonKaen province). Specimens were from children who had diarrhea (three or more loose watery stools per day) and moderate to severe dehydration with or without fever and vomiting. The study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine of Chulalongkorn University (IRB number 261/64).

RV detection

Viral RNA was extracted from 200 μL of 10% weigh-to-volume stool suspension in phosphate buffered saline, which was clarified by centrifugation at 4,000 x g for 10 minutes. Automated RNA extraction using a magLEAD 12gC reagents and instrument was performed according to the manufacturer's instructions (Precision System Science, Chiba, Japan). One-step real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed as previously described [203]. Briefly, viral RNA was denatured at 97°C for 5 min, then iced. Using QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany) and an ABI viiA7 Real-Time PCR System (Applied Biosystems), detection of the partial NSP3 gene was performed in a 25 μL reaction volume with forward primer NVP3-F (5'-GACGGVGCRACTACATGGT-3'), reverse primer NVP3-R (5'-

GTCCAATTCATNCCTGGTGG-3'), and NVP3 probe (5' FAM-ATGAGCACAATAGTT (BHQ1) AAAAGCTAACACTGTCAA-(6-C_spacer)-3'). RT-PCR conditions were 50°C for 30 min., followed by 43 cycles of 94°C for 15 sec and 60°C for 1 min. Cycle threshold (Ct) value ≤36 was considered positive for RV.

Genotyping

RV-positive samples were subjected to the amplification of VP6, VP7, and VP4 genes by using SensiFAST one-step RT-PCR reagent (Bioline, London, UK). VP6 gene primers used were VP6-F1 and VP6-R1357 [204]. VP7 gene amplification was a multiplex PCR in which the first PCR utilized Gouvea's primers Beg 9 and End 9 [205] and the second PCR utilized Fujii's primer set [206]. VP4 gene amplification was also a multiplex PCR performed as previously described [207]. RV of known genotypes G1, G2, G3, equine-like G3 (eG3), G4, G8, G9 and P[4], P[6], P[8], P[9], and P[10] as determined from the extensively sequenced archived samples from our previous studies served as positive controls [36]. To amplify the P[5] genotype, con2 reverse primer paired with the newly design forward primer VP4_P5_F (5'- ACCAGGTGTCACATCAGAA-3') was used. Amplified PCR products were visualized by agarose gel electrophoresis, excised, and purified, and submitted for Sanger sequencing.

Remaining RV gene segments were amplified using consensus primers and SuperScript III one-step RT-PCR kit (Invitrogen, USA) as previously described [204]. Reverse-transcription conditions were 45°C for 30 min followed by 94°C for 2 min. PCR conditions were 40 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 68°C for 1 min/kb. Amplicons were treated with ExoSAP (GE Healthcare, USA) and Sanger-sequenced. Genotyping of all 11 gene segments was performed using the Rotavirus A Genotype Determination available through the Virus Pathogen Resource (ViPR) website https://www.viprbrc.org/brc/rvaGenotyper.spg) [75]. All sequences were deposited in the GenBank database under the accession numbers ON058284-ON058286, ON191607-ON191626, and ON206900-ON206970.

Sequence analysis

Nucleotide sequences of individual segments were aligned with the RV reference sequences using ClustalW. Maximum-likelihood phylogenetic trees were constructed with the best substitution models determined based on the corrected Bayesian information criterion value and implemented in MEGA7 [208, 209]. Tree robustness was determined by bootstrapping of 1,000 replicates with values >70% considered significant.

Results

RV infection

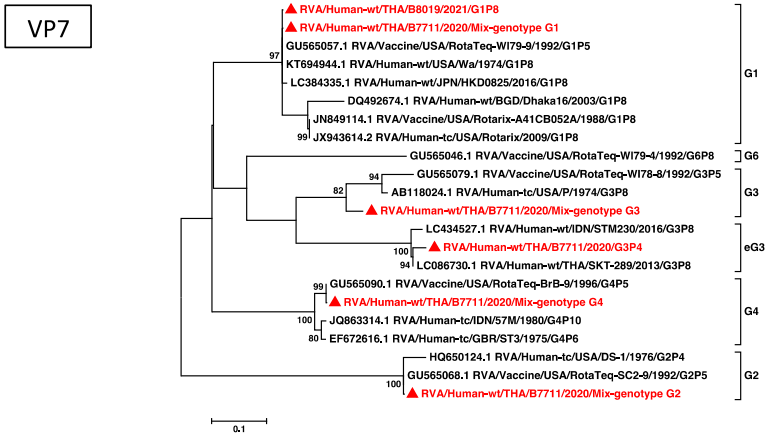
From 257 pediatric diarrhea samples submitted over the two calendar years, 25 samples (9.7%) tested positive for RV by real-time RT-PCR. After genotyping, samples either possessed a single genotype (19/25, 76%) or showed evidence of multiple RV co-infections (6/25, 24%). The most prevalent single-infection genotype was G3P[8] (12/19, 63.15%), followed by G1P[8] and G8P[8] (2/19 each, 10.52%). The remaining three were G2P[4], G9P[8], and G3P[x]. Among multiply infected RV samples, all were equine-G3 (eG3) with additional VP7 gene segments including G1, G2, G3, and G4. Five out of six multiple infections possessed VP4 of genotype P[8] (Table 5).

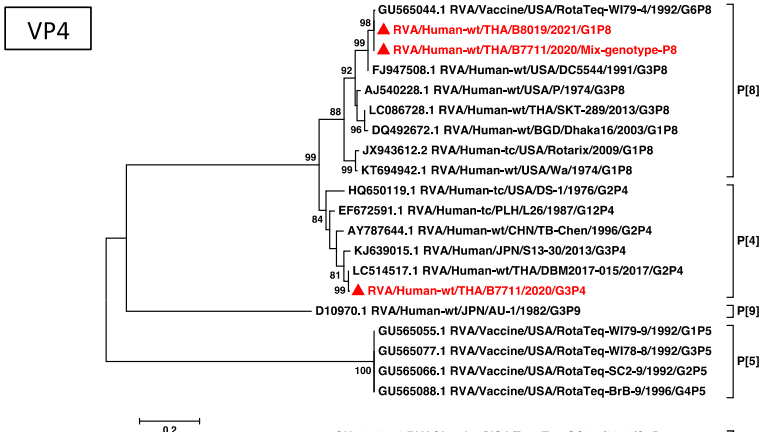
Table 5. **Multiple RV co-infections detected in diarrhea samples.**

Sample	VP7	VP4
B7469	G1, eG3	P[8]
B7521	G1, eG3	P[8]
B7731	G1, eG3	P[8]
B7711	G1, G2, G3, eG3, G4	P[4], P[5], P[8]
B7771	G3, eG3	P[8]
B7794	G2, eG3	P[4]

Genome analyses of RV strains of vaccine origin

Nucleotide sequences of two RV-positive samples (B7711 and B8019) showed remarkable resemblance to those of the parental vaccine strains in RotaTeq. Phylogenetic analysis demonstrated that all of the structural and non-structural gene segments in these samples clustered with RotaTeq vaccine strains (Figs 6 and 7). Several VP7 sequences were identified in B7711, including those very similar to the G1, G2, G3, G4 vaccine strains in RotaTeq with an additional sequence showing similarity to the eG3 of wild-type RV strain (Fig 6 and Table 6). Moreover, two sequences of VP4 identified in B7711 were RotaTeq P[5] and P[8], with an additional non-vaccine P[4] (>99% nucleotide identities). B7711 also possessed an NSP1 gene segment of genotypes A2 in addition to the vaccine A3 (Fig 7). Meanwhile, the VP7 and VP4 sequences of B8019 were genetically closest to G1 and P[8] of RotaTeq vaccine strains (>99.5% nucleotide identities). Both B7711 and B8019 shared identical nucleotide sequences to the NSP1 and NSP4 genes of the parental vaccine strains.


VP7
RVA/Human-wt/THA/B8019/2021/G1P8
RVA/Human-wt/THA/B7711/2020/Mix-genotype G1
GU565057.1 RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P5
KT694944.1 RVA/Human-wt/USA/Wa/1974/G1P8
LC384335.1 RVA/Human-wt/JPN/HKD0825/2016/G1P8
DQ492674.1 RVA/Human-wt/BGD/Dhaka16/2003/G1P8
JN849114.1 RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P8
JX943614.2 RVA/Human-tc/USA/Rotarix/2009/G1P8
G1
GU565046.1 RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P8
G6
GU565079.1 RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P5
AB118024.1 RVA/Human-tc/USA/P/1974/G3P8
RVA/Human-wt/THA/B7711/2020/Mix-genotype G3
G3
LC434527.1 RVA/Human-wt/IDN/STM230/2016/G3P8
RVA/Human-wt/THA/B7711/2020/G3P4
LC086730.1 RVA/Human-wt/THA/SKT-289/2013/G3P8
eG3
GU565090.1 RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P5
RVA/Human-wt/THA/B7711/2020/Mix-genotype G4
JQ863314.1 RVA/Human-tc/IDN/57M/1980/G4P10
EF672616.1 RVA/Human-tc/GBR/ST3/1975/G4P6
G4
HQ650124.1 RVA/Human-tc/USA/DS-1/1976/G2P4
GU565068.1 RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P5
RVA/Human-wt/THA/B7711/2020/Mix-genotype G2
G2
0.1


VP4
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RVA/Human-wt/THA/B8019/2021/G1P8
RVA/Human-wt/THA/B7711/2020/Mix-genotype-P8
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AJ540228.1 RVA/Human-wt/USA/P/1974/G3P8
LC086728.1 RVA/Human-wt/THA/SKT-289/2013/G3P8
DQ492672.1 RVA/Human-wt/BGD/Dhaka16/2003/G1P8
JX943612.2 RVA/Human-tc/USA/Rotarix/2009/G1P8
KT694942.1 RVA/Human-wt/USA/Wa/1974/G1P8
P[8]
HQ650119.1 RVA/Human-tc/USA/DS-1/1976/G2P4
EF672591.1 RVA/Human-tc/PLH/L26/1987/G12P4
AY787644.1 RVA/Human-wt/CHN/TB-Chen/1996/G2P4
KJ639015.1 RVA/Human/JPN/S13-30/2013/G3P4
LC514517.1 RVA/Human-wt/THA/DBM2017-015/2017/G2P4
RVA/Human-wt/THA/B7711/2020/G3P4
P[4]
D10970.1 RVA/Human-wt/JPN/AU-1/1982/G3P9
P[9]
GU565055.1 RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P5
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P[5]
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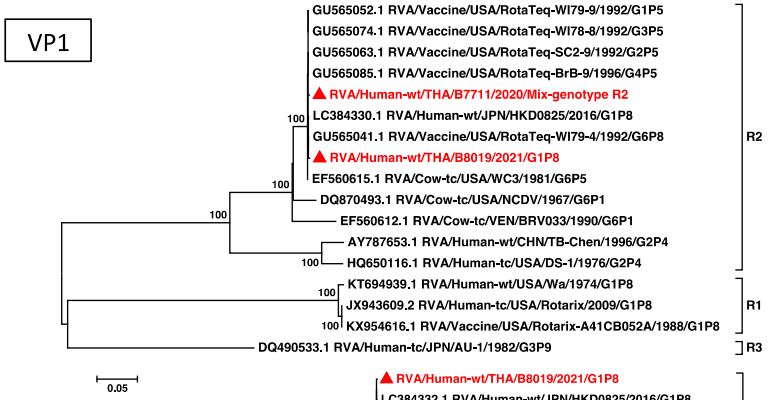
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GU565063.1 RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P5
GU565085.1 RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P5
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RVA/Human-wt/THA/B8019/2021/G1P8
EF560615.1 RVA/Cow-tc/USA/WC3/1981/G6P5
DQ870493.1 RVA/Cow-tc/USA/NCDV/1967/G6P1
EF560612.1 RVA/Cow-tc/VEN/BRV033/1990/G6P1
AY787653.1 RVA/Human-wt/CHN/TB-Chen/1996/G2P4
HQ650116.1 RVA/Human-tc/USA/DS-1/1976/G2P4
R2
KT694939.1 RVA/Human-wt/USA/Wa/1974/G1P8
JX943609.2 RVA/Human-tc/USA/Rotarix/2009/G1P8
KX954616.1 RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P8
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R3
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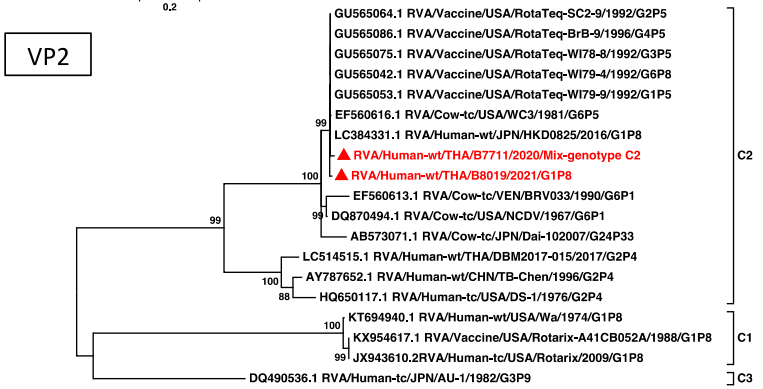
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GU565075.1 RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P5
GU565042.1 RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P8
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RVA/Human-wt/THA/B7711/2020/Mix-genotype C2
RVA/Human-wt/THA/B8019/2021/G1P8
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DQ870494.1 RVA/Cow-tc/USA/NCDV/1967/G6P1
AB573071.1 RVA/Cow-tc/JPN/Dai-102007/G24P33
LC514515.1 RVA/Human-wt/THA/DBM2017-015/2017/G2P4
AY787652.1 RVA/Human-wt/CHN/TB-Chen/1996/G2P4
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C2
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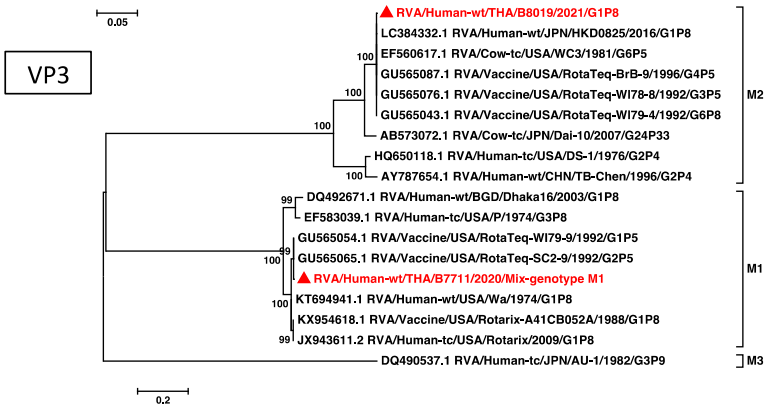
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M3
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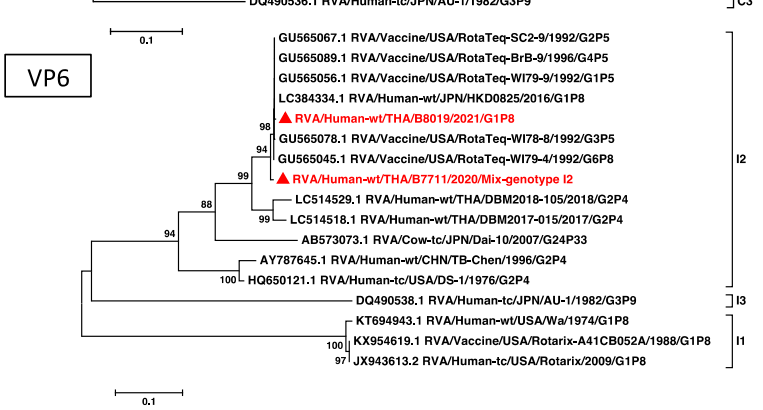
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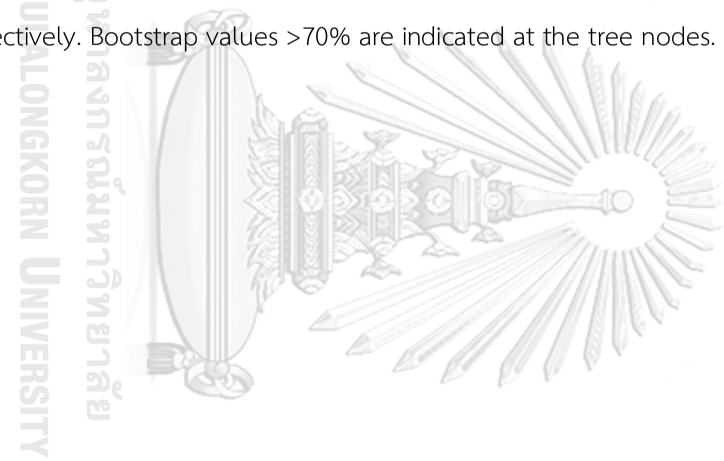
RVA/Human-wt/THA/B8019/2021/G1P8
GU565078.1 RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P5
GU565045.1 RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P8
RVA/Human-wt/THA/B7711/2020/Mix-genotype I2
LC514529.1 RVA/Human-wt/THA/DBM2018-105/2018/G2P4
LC514518.1 RVA/Human-wt/THA/DBM2017-015/2017/G2P4
AB573073.1 RVA/Cow-tc/JPN/Dai-10/2007/G24P33
AY787645.1 RVA/Human-wt/CHN/TB-Chen/1996/G2P4
HQ650121.1 RVA/Human-tc/USA/DS-1/1976/G2P4
I2
DQ490538.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
I3
KT694943.1 RVA/Human-wt/USA/Wa/1974/G1P8
KX954619.1 RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P8
JX943613.2 RVA/Human-tc/USA/Rotarix/2009/G1P8
I1
0.1

Figure 6. **Phylogenetic analysis of the nucleotide sequences of RV structural protein genes.**

Comparisons of the parental vaccine strains in RotaTeq and the vaccine-derived strains in this study (noted with triangles and in red) are shown on the trees. Nucleotide sequence lengths used in the maximum-likelihood phylogenetic analysis for VP1, VP2, VP3, VP4, VP6, and VP7 genes were 3019, 1112, 1092, 220, 1138, and 138 base pairs, respectively. Bootstrap values >70% are indicated at the tree nodes. Scale bars represent substitutions per nucleotide.



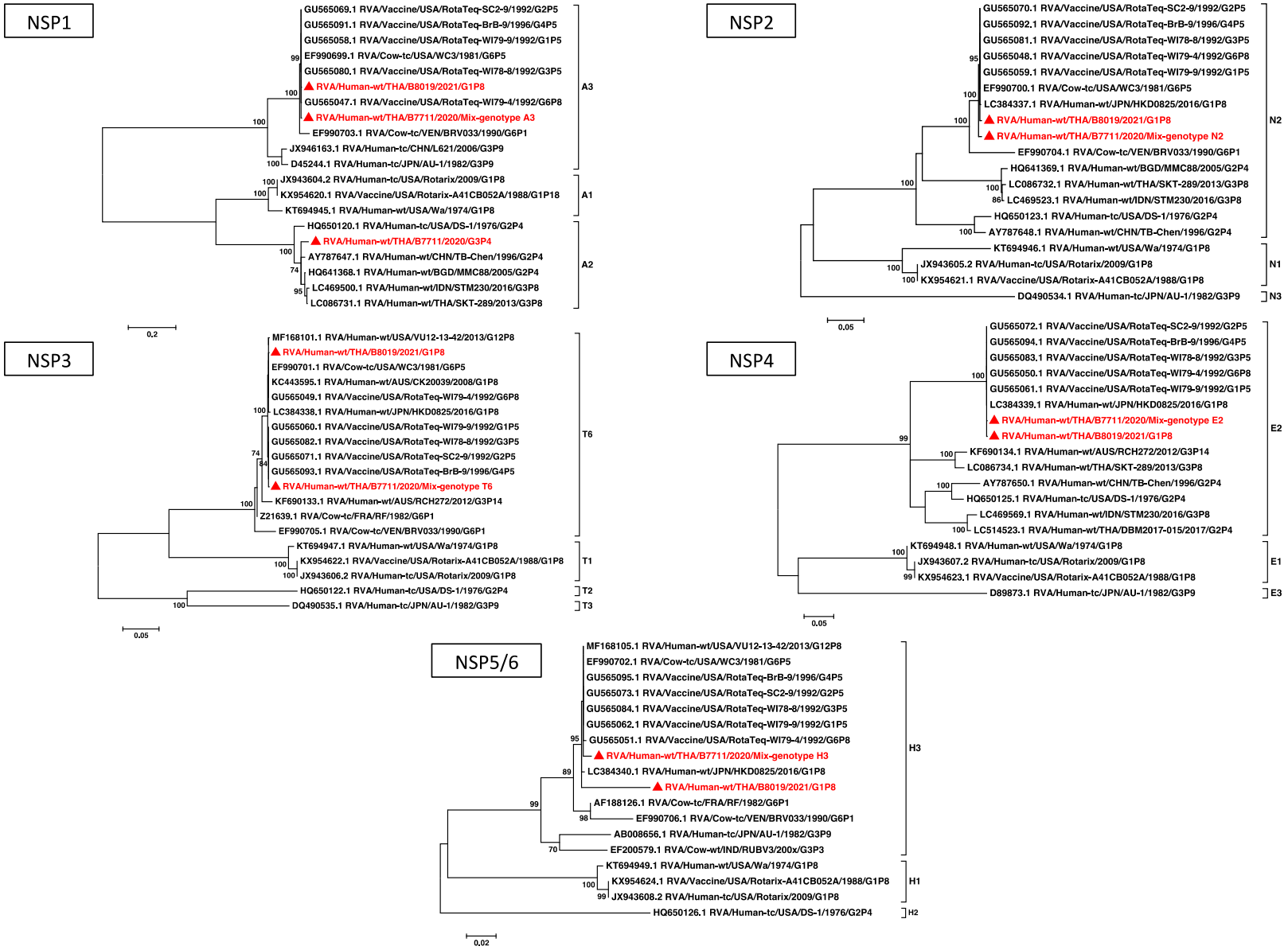
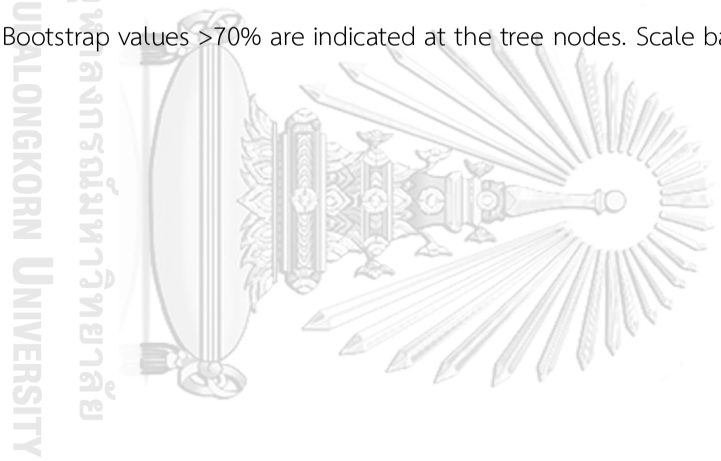

NSP1
GU565069.1 RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P5
GU565091.1 RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P5
GU565058.1 RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P5
EF990699.1 RVA/Cow-tc/USA/WC3/1981/G6P5
GU565080.1 RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P5
RVA/Human-wt/THA/B8019/2021/G1P8
GU565047.1 RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P8
RVA/Human-wt/THA/B7711/2020/Mix-genotype A3
EF990703.1 RVA/Cow-tc/VEN/BRV033/1990/G6P1
JX946163.1 RVA/Human-tc/CHN/L621/2006/G3P9
D45244.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
JX943604.2 RVA/Human-tc/USA/Rotarix/2009/G1P8
KX954620.1 RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P18
KT694945.1 RVA/Human-wt/USA/Wa/1974/G1P8
HQ650120.1 RVA/Human-tc/USA/DS-1/1976/G2P4
RVA/Human-wt/THA/B7711/2020/G3P4
AY787647.1 RVA/Human-wt/CHN/TB-Chen/1996/G2P4
HQ641368.1 RVA/Human-wt/BGD/MMC88/2005/G2P4
LC469500.1 RVA/Human-wt/IDN/STM230/2016/G3P8
LC086731.1 RVA/Human-wt/THA/SKT-289/2013/G3P8
A3
A1
A2
0.2
NSP2
GU565070.1 RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P5
GU565092.1 RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P5
GU565081.1 RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P5
GU565048.1 RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P8
GU565059.1 RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P5
EF990700.1 RVA/Cow-tc/USA/WC3/1981/G6P5
LC384337.1 RVA/Human-wt/JPN/HKD0825/2016/G1P8
RVA/Human-wt/THA/B8019/2021/G1P8
RVA/Human-wt/THA/B7711/2020/Mix-genotype N2
EF990704.1 RVA/Cow-tc/VEN/BRV033/1990/G6P1
HQ641369.1 RVA/Human-wt/BGD/MMC88/2005/G2P4
LC086732.1 RVA/Human-wt/THA/SKT-289/2013/G3P8
LC469523.1 RVA/Human-wt/IDN/STM230/2016/G3P8
HQ650123.1 RVA/Human-tc/USA/DS-1/1976/G2P4
AY787648.1 RVA/Human-wt/CHN/TB-Chen/1996/G2P4
KT694946.1 RVA/Human-wt/USA/Wa/1974/G1P8
JX943605.2 RVA/Human-tc/USA/Rotarix/2009/G1P8
KX954621.1 RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P8
DQ490534.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
N2
N1
N3
0.05
NSP3
MF168101.1 RVA/Human-wt/USA/VU12-13-42/2013/G12P8
RVA/Human-wt/THA/B8019/2021/G1P8
EF990701.1 RVA/Cow-tc/USA/WC3/1981/G6P5
KC443595.1 RVA/Human-wt/AUS/CK20039/2008/G1P8
GU565049.1 RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P8
LC384338.1 RVA/Human-wt/JPN/HKD0825/2016/G1P8
GU565060.1 RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P5
GU565082.1 RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P5
GU565071.1 RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P5
GU565093.1 RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P5
RVA/Human-wt/THA/B7711/2020/Mix-genotype T6
KF690133.1 RVA/Human-wt/AUS/RCH272/2012/G3P14
Z21639.1 RVA/Cow-tc/FRA/RF/1982/G6P1
EF990705.1 RVA/Cow-tc/VEN/BRV033/1990/G6P1
KT694947.1 RVA/Human-wt/USA/Wa/1974/G1P8
KX954622.1 RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P8
JX943606.2 RVA/Human-tc/USA/Rotarix/2009/G1P8
HQ650122.1 RVA/Human-tc/USA/DS-1/1976/G2P4
DQ490535.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
T6
T1
T2
T3
0.05
NSP4
GU565072.1 RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P5
GU565094.1 RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P5
GU565083.1 RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P5
GU565050.1 RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P8
GU565061.1 RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P5
LC384339.1 RVA/Human-wt/JPN/HKD0825/2016/G1P8
RVA/Human-wt/THA/B7711/2020/Mix-genotype E2
RVA/Human-wt/THA/B8019/2021/G1P8
KF690134.1 RVA/Human-wt/AUS/RCH272/2012/G3P14
LC086734.1 RVA/Human-wt/THA/SKT-289/2013/G3P8
AY787650.1 RVA/Human-wt/CHN/TB-Chen/1996/G2P4
HQ650125.1 RVA/Human-tc/USA/DS-1/1976/G2P4
LC469569.1 RVA/Human-wt/IDN/STM230/2016/G3P8
LC514523.1 RVA/Human-wt/THA/DBM2017-015/2017/G2P4
KT694948.1 RVA/Human-wt/USA/Wa/1974/G1P8
JX943607.2 RVA/Human-tc/USA/Rotarix/2009/G1P8
KX954623.1 RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P8
D89873.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
E2
E1
E3
0.05
NSP5/6
MF168105.1 RVA/Human-wt/USA/VU12-13-42/2013/G12P8
EF990702.1 RVA/Cow-tc/USA/WC3/1981/G6P5
GU565095.1 RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P5
GU565073.1 RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P5
GU565084.1 RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P5
GU565062.1 RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P5
GU565051.1 RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P8
RVA/Human-wt/THA/B7711/2020/Mix-genotype H3
LC384340.1 RVA/Human-wt/JPN/HKD0825/2016/G1P8
RVA/Human-wt/THA/B8019/2021/G1P8
AF188126.1 RVA/Cow-tc/FRA/RF/1982/G6P1
EF990706.1 RVA/Cow-tc/VEN/BRV033/1990/G6P1
AB008656.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
EF200579.1 RVA/Cow-wt/IND/RUBV3/200x/G3P3
KT694949.1 RVA/Human-wt/USA/Wa/1974/G1P8
KX954624.1 RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P8
JX943608.2 RVA/Human-tc/USA/Rotarix/2009/G1P8
HQ650126.1 RVA/Human-tc/USA/DS-1/1976/G2P4
H3
H1
H2
0.02

Figure 7. **Phylogenetic analysis of the nucleotide sequences of RV non-structural protein genes.**

Comparisons of the parental vaccine strains in RotaTeq and the vaccine-derived strains in this study (noted with triangles and in red) are shown on the trees. Nucleotide sequence lengths used in the maximum-likelihood phylogenetic analysis for NSP1, NSP2, NSP3, NSP4, and NSP5 genes were 1271, 894, 912, 505, and 554 base pairs, respectively. Bootstrap values >70% are indicated at the tree nodes. Scale bars represent substitutions per nucleotide.



Clinical characteristics associated with B7711 and B8019

B7711 was from a 7 month-old girl who had previously received 3 doses of RotaTeq. Fifteen days after the third dose, she presented with diarrhea, fever, and mild dehydration. Co-morbidity included laboratory-confirmed norovirus infection.

B8019 was from a 3-month-old boy who had previously received a single dose of RotaTeq. Thirty-one days post-vaccination, he presented with watery and bloody diarrhea, fever, mild dehydration, and lethargy. This sample tested negative for norovirus.

Table 6**. Genome constellation of RV in RotaTeq compared to Thai strains.**

Samples	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7A[5]	G1	P[5]	I2	R2	C2	M1	A3	N2	T6	E2	H3
RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7A[5]	G2	P[5]	I2	R2	C2	M1	A3	N2	T6	E2	H3
RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P7A[5]	G3	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P7A[5]	G4	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]	G6	P[8]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Human-wt/THA/B7711/2020/Mix-genotype	G1/ G2/ G3/eG3/G4	P[4]/ P[5]/ P[8]	I2	R2	C2	M1	A2/A3	N2	T6	E2	H3
RVA/Human-wt/THA/B8019/2021/G1P[8]	G1	P[8]	I2	R2	C2	M2	A3	N2	T6	E2	H3

Discussion

RV infection remains a major cause of childhood diarrhea particularly in regions without universal RV vaccination. Prior to February 2020, Thai parents wanting to vaccinate their children sought out RV vaccines at private hospitals. Thus, knowledge regarding vaccine-associated RV shedding following routine vaccination in Thailand is currently absent. In this study, we surveyed diarrheal samples for RV during 2020 and 2021 at the time of heighten coronavirus infection and found that approximately 10% of pediatric diarrhea was attributable to RV infection. This prevalence is lower than previous years, possibly because of social measures implemented by the Thai government to mitigate coronavirus transmission and an early impact of RV vaccine inclusion in Thailand national immunization program [37, 202]. It was not surprising that G3P[8] was the most frequent genotype detected because this strain has predominated in Thailand in the last few years [46]. More importantly, two samples from babies with diarrhea demonstrated evidence of vaccine-derived RV infection, including one sample with possible co-infection with a wild-type RV at around the time of vaccination.

The use of conventional multiplex RT-PCR in our study revealed many mixed infections with more than one RV strain in each sample (24%). This is a novel finding compared to our previous studies in which we mostly relied on a simple RT-PCR and had resulted in the detection bias of a single RV strain per sample [36, 37]. Although time-consuming and often requires confirmatory Sanger sequencing analysis, it has the potential to reveal the burden of multiple RV infections and tracing the emergence of viral reassortants. Finding of frequent RV mixed infection is not new as it has previously been reported in Zambia and elsewhere [210]. The major disadvantage in using

multiplex RT-PCR is that genotype-specific forward primers bind at different positions. The resulting amplified gene fragments are therefore of different lengths and require an additional phylogenetic tree analysis to show genetic clusters (S1 Fig).

Vaccine recipients in this study developed diarrhea approximately two and four weeks after RotaTeq vaccination, which were within the time span previously reported [198]. A post-implementation study also reported viral shedding among vaccinated children within 1-4 weeks after any vaccine dose [199]. Large studies from countries with longstanding RV universal vaccination have shown that symptomatic RV infection as a result of routine vaccination occur at the prevalence of 46.7% in Australia [211], 21.4% in the USA [196], and 13.6% in South Korea [212]. It is interesting to note that viral shedding after RotaTeq vaccination was reportedly lower in viral loads compared to Rotarix [213] since the former replicates poorly in humans due to the bovine parental backbone strain of the vaccine [193]. While we could confidently conclude that B8019 possessed genome constellation G1-P[8]-I2-R2-C2-M2-A3-N2-T6-E2-H3, multiple vaccine-like RV strains detected in B7711 combined with probable wild-type RV co-infection prevents us from assigning definitive constellation. Consistent with our study, multiple vaccine-like RV strains were also found in an Australian study in children with underlying medical condition [214]. One possibility for the derivation of G1P[8] in B8019 is the dual reassortment between two parental strains from RotaTeq. Vaccine-derived reassortant G1P[8] has been described in patients in the USA, Australia, and Finland following RotaTeq vaccination [35]. In contrast, VP7 of genotype equine-like G3, VP4 of genotype P[4], and NSP1 of genotype A2 are not represented in RotaTeq and were likely from

a wild-type RV. Concurrent infection with the vaccine and wild-type RV strains have been reported in Japan [215] and South Korea [212].

This study has several limitations. The majority of the diarrhea samples (90%) tested negative for RV even though we used a standardized assay developed by the U.S. Centers for Disease Control and Prevention [203]. Due to the coronavirus pandemic and the prolonged closures of schools, diarrhea samples sent to us for testing were very low compared to past years. This in part hinders the investigation of diarrhea associated with post-vaccination, and the true rate of symptomatic diarrhea or asymptomatic shedding of vaccine-derived RV may be difficult to determine as there is no current epidemiological surveillance program to systematically assess this. Nevertheless, the long overdue introduction of universal RV vaccination in Thailand is expected to reduce the incidence of RV-associated diarrhea requiring hospitalization among very young children.

Conclusions

Findings in this study demonstrated possible vaccine-derived RV shedding in Thai children with diarrhea, and the observed wild-type RV infection concurrent with recent RotaTeq vaccination. RV infection is predominated by the G3P[8] strain. Potential RV shedding among vaccine recipients will require monitoring, especially in household with many children or daycare to mitigate potential horizontal transmission of vaccine-derived RV.

CHAPTER V

Diverse human and bat-like rotavirus G3 strains circulating in suburban Bangkok

Publication

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Abstract

Although rotavirus vaccines are available in many parts of the world and are effective in reducing the overall incidence of rotavirus infection, it remains a major cause of diarrhea in less-developed countries. Among various rotavirus group A (RVA) strains, the increasingly common genotype G3 (defined by the VP7 gene) has been identified in both humans and animals. Our previous epidemiological surveillance in Bangkok found several unusual non-vaccine-like G3 strains in patients with diarrhea. In this study, we sequenced and characterized the genomes of seven of these G3 strains, which formed combinations with genotypes P[4], P[6], P[9], and P[10] (defined by the VP4 gene). Interestingly, we identified a bat-like RVA strain with the genome constellation G3-P[10]-I3-R3-C3-M3-A9-N3-T3-E3-H6, which has not been previously reported in the literature. The amino acid residues deduced from the nucleotide sequences of our G3 strains differed at the antigenic epitopes to those of the VP7 capsid protein of the G3 strain in RotaTeq vaccine. Although it is not unusual for the segmented genomes of RVA to reassort and give rise to emerging novel strains, the atypical G3 strains identified in this study suggest possible animal-to-human RVA zoonotic spillover even in urban areas.

Introduction

Rotavirus is a major cause of viral diarrhea in very young children. Infection accounts for approximately 120,000 deaths annually in children younger than 5 years of age, the majority of whom live in developing countries [3]. There are designated 9 distinct rotavirus species (A-D and F-J) and 2 putative RV species K and L, of which rotavirus group A (RVA) most often cause infection in humans [21, 22].

RVA belongs to the family *Reoviridae* and possesses segmented double-stranded RNA genome [216]. The virion is non-enveloped and has a triple-layered capsid. The 11 genomic RNA segments encode viral structural (VP1 to VP4, VP6, and VP7) and non-structural (NSP1 to NSP5) proteins [217]. The Rotavirus Classification Working Group (RCWG) designates the 11 RV genome segments encoding VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 into the corresponding genotype constellation Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, respectively [26]. To date, there are 41 G, 57 P, 31 I, 27 R, 23 C, 23 M, 38 A, 27 N, 27 T, 31 E, and 27 H genotypes among the RVA identified in human and animal species worldwide [23]. For most epidemiological surveillance of rotavirus, RVA genotype is often described by identifying the sequence of the VP7 glycoprotein (G) and VP4 protease-sensitive (P) protein [20].

RVA segmented genomes are amenable to reassortment, often resulting in strains with new genetic and antigenic properties [16, 218]. Due to the diversity of species RVA can infect, spillover infection of animal RVA into humans is common. Detection of such infection often requires extensive sequencing and determination of the viral genome constellation [20, 219-222], which can

help determine the shared origin and evolutionary relationships among different RVA strains [223].

RVA of genotype G3 is one of the frequently detected strains worldwide [224-226]. The broad host range for G3 including feline, canine, equine, porcine, lapine, bovine, rats, rhesus, and bats has resulted in the occasional detection of animal-like RVA infection in patients with diarrhea [222, 227-232]. Although G3 is often found in combination with P[8], P[4], and P[6], pairings with P[9] and P[10] are less frequently reported [115, 225, 229, 233-236]. Previously, we conducted surveillances of RVA infection in Thailand from 2015 to 2019 and found that G3 strains were most predominantly detected in adults and children with viral diarrhea [36, 37, 46]. Of interests are several atypical G3 strains not associated with the rotavirus vaccine strain, which could have arisen from zoonotic transmission. In this study, we characterized seven uncommon G3 strains from children and adults with RVA infection living in Bangkok.

Materials and Methods

RVA genotyping

Among the RVA strains identified in patients with diarrhea during 2016-2018 [36, 37], seven unusual G3 strains initially determined based on the partial nucleotide sequence information were examined (strains B5383, B5356, B4401, B5368, B2682, B4684, and B5662). Archived viral RNA samples were subjected to conventional reverse-transcription polymerase chain reaction (RT-PCR) as previously described [204], including the use of additional primers to amplify the VP4 gene of P[10] (S1 Table). The PCR conditions were 40 cycles of denaturation at 94°C for 30 sec., annealing at 56°C for 45 sec., and extension at 72°C for 2 min. Amplicons were treated with ExoSAP (GE Healthcare, USA) prior to Sanger sequencing. Nucleotide sequences were analyzed using BioEdit [237] and subjected to BLAST search to yield percent nucleotide sequence identities (www.ncbi.nlm.nih.gov). Genotyping of all 11 gene segments was performed using the Rotavirus A Genotype Determination available through the Virus Pathogen Resource (ViPR) website https://www.viprbrc.org/brc/rvaGenotyper.spg) [75]. In addition to the previously deposited nucleotide sequences in the GenBank database, additional sequences were submitted under the accession numbers MW720858-MW720877, OK243970-OK243979, and OK244000-OK244038.

Nucleotide sequence analysis

Gene segments were aligned with the RVA reference sequences by using ClustalW. Maximum-likelihood phylogenetic trees were constructed with the best substitution models determined based on the corrected Bayesian information criterion value implemented in MEGA7 [208, 209]. The models used in this study were General Time Reversible (GTR) + gamma distributed (G) + invariable sites (I) (for VP1, VP2, VP3, and NSP1), Tamura 3-parameter (T92) + G + I (for VP4, VP6, VP7, NSP2, NSP3, and NSP5) and T92 + G (for NSP4). Tree robustness was determined by bootstrapping of 1,000 replicates with values >70% considered significant.

Amino acid residue analysis

Deduced amino acid residues from the nucleotide-sequenced strains were aligned with those of the RVA vaccine G3 in RotaTeq. Changes were mapped onto the published structure of trimeric VP7 (Protein Data Bank number 3FMG) using PyMOL software.

Results

Seven G3 strains (B5383, B5356, B4401, B5368, B2682, B4684, and B5662) in various combinations with P[4], P[6], P[9], and P[10] were from patients 2 to 49 years of age of both genders, only two of whom are children (<4 years old) (Table 7). These samples were from infection detected from December to May of each year, which coincide with the typical annual RVA season in Thailand.

Table 7. **Description of the RVA samples in this study.**

Strain designation	Collection date	Gender	Age
RVA/Human-wt/THA/B5383/2018/G3P[4]	06/03/18	F	34 yr 11 mo
RVA/Human-wt/THA/B5356/2018/G3P[4]	02/03/18	F	29 yr 6 mo
RVA/Human-wt/THA/B4401/2017/G3P[6]	23/12/17	M	3 yr 11 mo
RVA/Human-wt/THA/B5368/2018/G3P[6]	05/03/18	F	49 yr 4 mo
RVA/Human-wt/THA/B2682/2016/G3P[9]	24/03/16	M	2 yr
RVA/Human-wt/THA/B4684/2018/G3P[10]	25/01/18	M	34 yr 7 mo
RVA/Human-wt/THA/B5662/2018/G3P[10]	03/05/18	F	44 yr 10 mo

F, female; M, male; yr, years; mo, months.

RVA genome constellation

To further characterize these atypical G3 strains, we determined the near-complete nucleotide sequences of all 11 gene segments. The genome constellations of the two G3P[4] (B5383 and B5356) and two G3P[6] (B4401 and B5368) mostly resembled the prototypic DS-1 (I2-R2-C2-M2-A2-N2-T2-E2-H2 (Table 8). Our G3P[6] strains, however, had identical genome constellation as RVA/Human-wt/IDN/STM182/2016/G3P[6] identified in Indonesia in 2016. Meanwhile, the G3P[9] mirrored the prototypic AU-1 in all gene segments except NSP5. Although 8 gene segments in the two G3P[10] (B4684 and B5662) shared similarities with AU-1, neither strains were identical to any established reference strains. When compared to global strains, however, they were genotypically identical in all gene segments except VP6 to the RVA/Bat-tc/CHN/MYAS33/2013/G3P[10] and RVA/Human-wt/THA/MS2015-1-0001/2015/G3P[10], of which the latter was identified in 2015 from a patient who lived in northern Thailand.

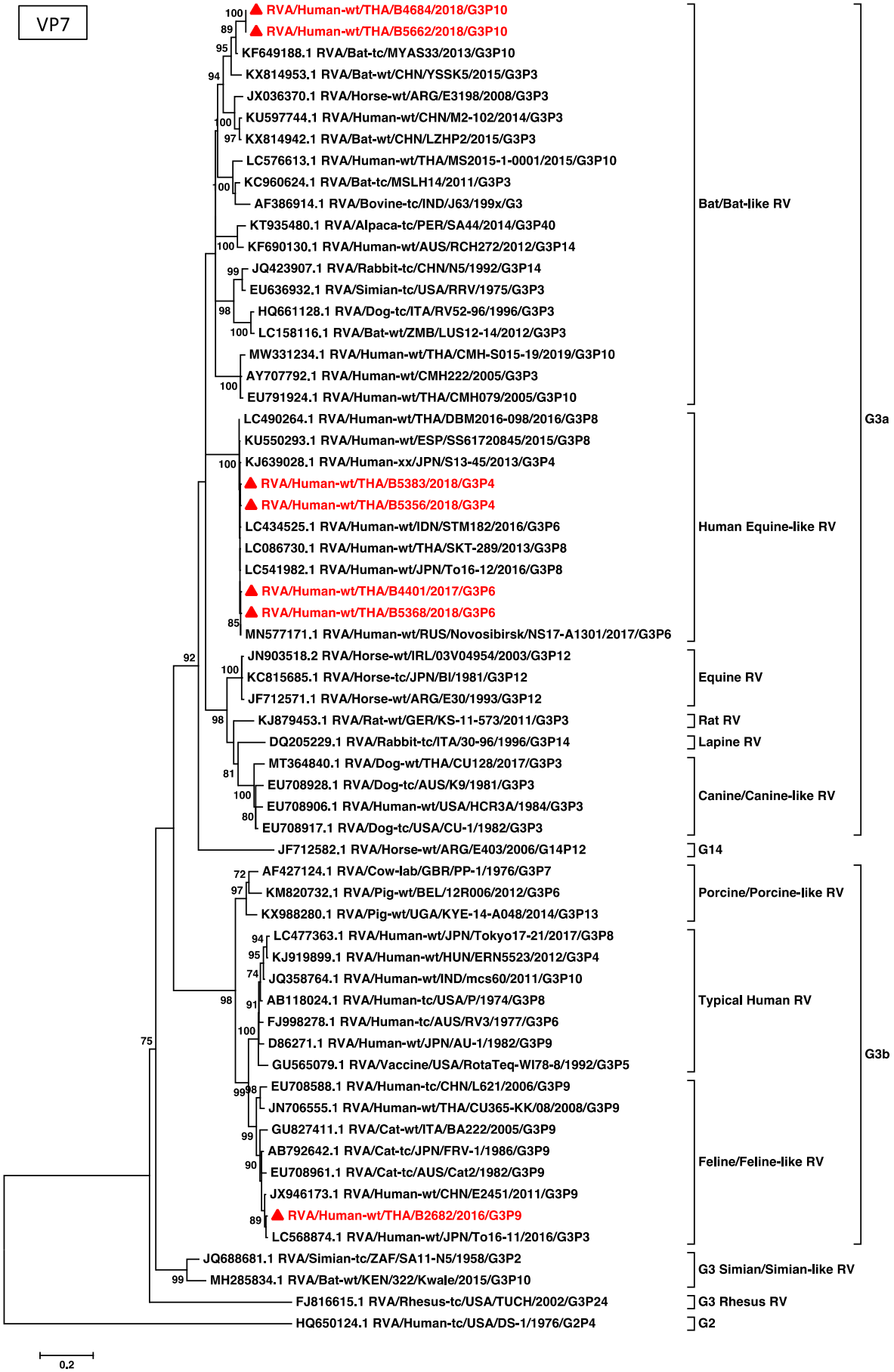
Table 8. **Genome constellations of RVA G3 characterized in this study (in italics) compared to several RCWG reference (in bold) and global strains.**

Strain Name	Genotypes										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Human-tc/USA/DS-1/1976/G2P[4]	**G2**	**P[4]**	**I2**	**R2**	**C2**	**M2**	**A2**	**N2**	**T2**	**E2**	**H2**
RVA/Human-wt/THA/B5383/2018/G3P[4]	G3	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/THA/B5356/2018/G3P[4]	G3	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/THA/B4401/2017/G3P[6]	G3	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/THA/B5368/2018/G3P[6]	G3	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/IDN/STM182/2016/G3P[6]	G3	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6]	G3	P[6]	I2	Rx	Cx	Mx	Ax	N2	Tx	E2	H2
RVA/Human-wt/JPN/AU-1/1982/G3P[9]	**G3**	**P[9]**	**I3**	**R3**	**C3**	**M3**	**A3**	**N3**	**T3**	**E3**	**H3**
RVA/Human-wt/THA/B2682/2016/G3P[9]	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
RVA/Human-wt/THA/B4684/2018/G3P[10]	G3	P[10]	I3	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Human-wt/THA/B5662/2018/G3P[10]	G3	P[10]	I3	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	G3	P[10]	I8	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Human-wt/THA/MS2015-1-0001/2015/G3P[10]	G3	P[10]	I8	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Human-wt/CHN/M2-102/2014/G3P[3]	G3	P[3]	I3	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Rhesus-tc/USA/TUCH/2002/G3P[24]	**G3**	**P[24]**	**I9**	**R3**	**C3**	**M3**	**A9**	**N1**	**T3**	**E3**	**H6**

Analyses using phylogenetic trees and pairwise comparisons

To examine the extent of how our G3 strains were similar to the reference and global RVA strains, we performed phylogenetic analyses for all of their 11 gene segments (Figs 8 and 9). The VP7 gene of our G3P[4] and G3P[6] strains clustered with human equine-like RVA, while the G3P[9] clustered with the feline-like RVA. Our two G3P[10] strains grouped with the bat-like MYAS33 strain. Meanwhile, our VP4 gene sequences were similar to various global strains. In particular, one of the two G3P[6] strains appeared closest to RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6], while both of our G3P[10] strains were very similar to the MS2015-1-0001 and MYAS33 strains.

Pairwise comparison of the nucleotide sequences of our G3P[4] and G3P[6] strains were ≥99% identical to the gene segments of global RVA strains (S2 Table). Our G3P[9] also shared >97% sequence identities with other previously described strains. In contrast, nucleotide identities for some of the G3P[10] gene segments were lower. While the VP6 gene of our G3P[10] strains were >98% identical to the M2-102 strain identified in China, the VP3 genes were only 88% identical to the closest known relative RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]. Moreover, the VP1 gene of G3P[10] strain B4684 (89%) was lower than B5662 (93%) when compared to the most similar MYAS33 strain.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LC576613.1 RVA/Human-wt/THA/MS2015-1-0001/2015/G3P10
KC960624.1 RVA/Bat-tc/MSLH14/2011/G3P3
AF386914.1 RVA/Bovine-tc/IND/J63/199x/G3
KT935480.1 RVA/Alpaca-tc/PER/SA44/2014/G3P40
KF690130.1 RVA/Human-wt/AUS/RCH272/2012/G3P14
JQ423907.1 RVA/Rabbit-tc/CHN/N5/1992/G3P14
EU636932.1 RVA/Simian-tc/USA/RRV/1975/G3P3
HQ661128.1 RVA/Dog-tc/ITA/RV52-96/1996/G3P3
LC158116.1 RVA/Bat-wt/ZMB/LUS12-14/2012/G3P3
MW331234.1 RVA/Human-wt/THA/CMH-S015-19/2019/G3P10
AY707792.1 RVA/Human-wt/CMH222/2005/G3P3
EU791924.1 RVA/Human-wt/THA/CMH079/2005/G3P10
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KU550293.1 RVA/Human-wt/ESP/SS61720845/2015/G3P8
KJ639028.1 RVA/Human-xx/JPN/S13-45/2013/G3P4
RVA/Human-wt/THA/B5383/2018/G3P4
RVA/Human-wt/THA/B5356/2018/G3P4
LC434525.1 RVA/Human-wt/IDN/STM182/2016/G3P6
LC086730.1 RVA/Human-wt/THA/SKT-289/2013/G3P8
LC541982.1 RVA/Human-wt/JPN/To16-12/2016/G3P8
RVA/Human-wt/THA/B4401/2017/G3P6
RVA/Human-wt/THA/B5368/2018/G3P6
MN577171.1 RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P6
JN903518.2 RVA/Horse-wt/IRL/03V04954/2003/G3P12
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KJ879453.1 RVA/Rat-wt/GER/KS-11-573/2011/G3P3
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LC477363.1 RVA/Human-wt/JPN/Tokyo17-21/2017/G3P8
KJ919899.1 RVA/Human-wt/HUN/ERN5523/2012/G3P4
JQ358764.1 RVA/Human-wt/IND/mcs60/2011/G3P10
AB118024.1 RVA/Human-tc/USA/P/1974/G3P8
FJ998278.1 RVA/Human-tc/AUS/RV3/1977/G3P6
D86271.1 RVA/Human-wt/JPN/AU-1/1982/G3P9
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EU708588.1 RVA/Human-tc/CHN/L621/2006/G3P9
JN706555.1 RVA/Human-wt/THA/CU365-KK/08/2008/G3P9
GU827411.1 RVA/Cat-wt/ITA/BA222/2005/G3P9
AB792642.1 RVA/Cat-tc/JPN/FRV-1/1986/G3P9
EU708961.1 RVA/Cat-tc/AUS/Cat2/1982/G3P9
JX946173.1 RVA/Human-wt/CHN/E2451/2011/G3P9
RVA/Human-wt/THA/B2682/2016/G3P9
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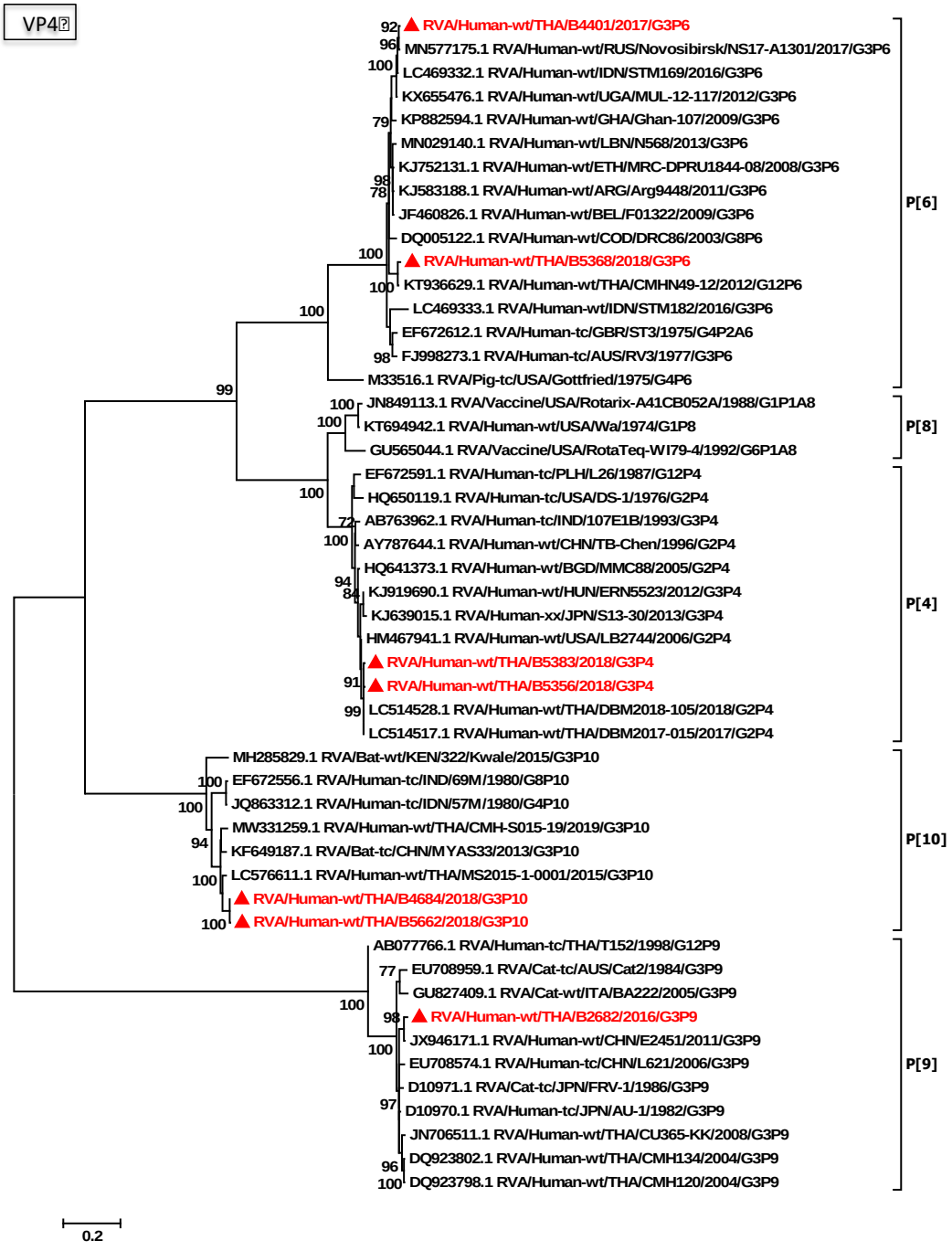
JQ688681.1 RVA/Simian-tc/ZAF/SA11-N5/1958/G3P2
MH285834.1 RVA/Bat-wt/KEN/322/Kwale/2015/G3P10
FJ816615.1 RVA/Rhesus-tc/USA/TUCH/2002/G3P24
HQ650124.1 RVA/Human-tc/USA/DS-1/1976/G2P4
Bat/Bat-like RV
Human Equine-like RV
Equine RV
Rat RV
Lapine RV
Canine/Canine-like RV
G14
Porcine/Porcine-like RV
Typical Human RV
Feline/Feline-like RV
G3 Simian/Simian-like RV
G3 Rhesus RV
G2
G3a
G3b
0.2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MN029140.1 RVA/Human-wt/LBN/N568/2013/G3P6
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P[6]
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KT694942.1 RVA/Human-wt/USA/Wa/1974/G1P8
GU565044.1 RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A8
P[8]
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LC514517.1 RVA/Human-wt/THA/DBM2017-015/2017/G2P4
P[4]
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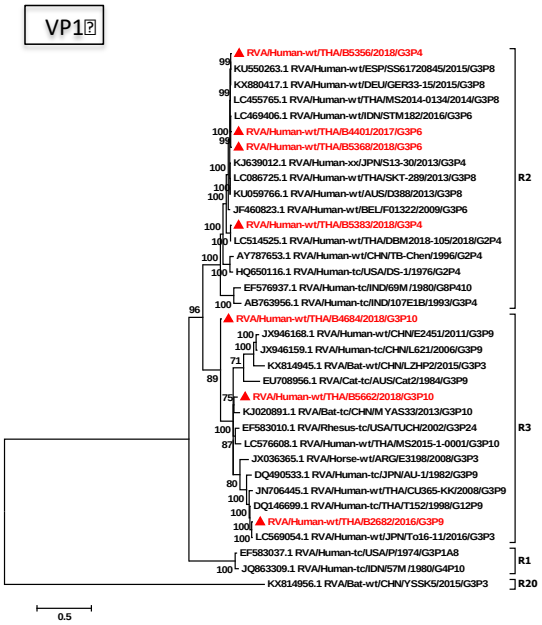
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RVA/Human-wt/THA/B5383/2018/G3P4
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R2
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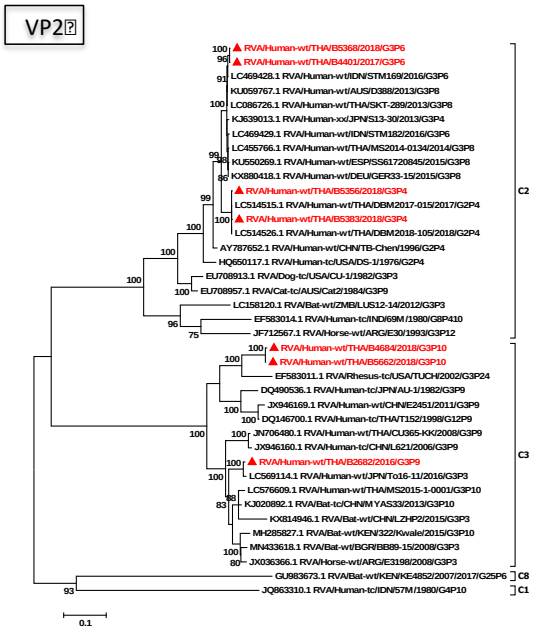
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MN433618.1 RVA/Bat-wt/BGR/BB89-15/2008/G3P3
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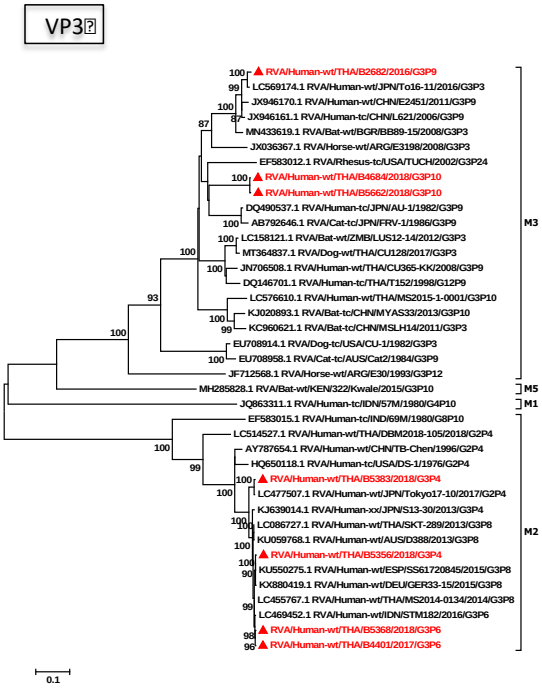
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MN433619.1 RVA/Bat-wt/BGR/BB89-15/2008/G3P3
JX036367.1 RVA/Horse-wt/ARG/E3198/2008/G3P3
EF583012.1 RVA/Rhesus-tc/USA/TUCH/2002/G3P24
RVA/Human-wt/THA/B4684/2018/G3P10
RVA/Human-wt/THA/B5662/2018/G3P10
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LC576610.1 RVA/Human-wt/THA/MS2015-1-0001/G3P10
KJ020893.1 RVA/Bat-tc/CHN/MYAS33/2013/G3P10
KC960621.1 RVA/Bat-tc/CHN/MSLH14/2011/G3P3
EU708914.1 RVA/Dog-tc/USA/CU-1/1982/G3P3
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M1
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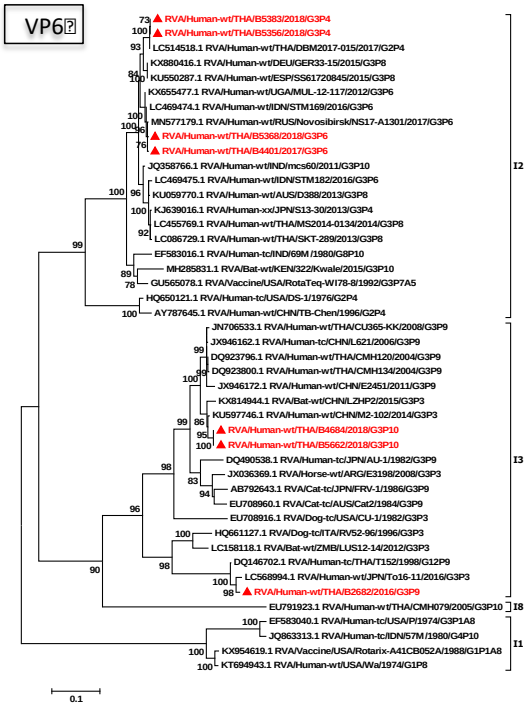
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LC158118.1 RVA/Bat-wt/ZMB/LUS12-14/2012/G3P3
DQ146702.1 RVA/Human-tc/THA/T152/1998/G12P9
LC568994.1 RVA/Human-wt/JPN/To16-11/2016/G3P3
RVA/Human-wt/THA/B2682/2016/G3P9
I3
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JQ863313.1 RVA/Human-tc/IDN/57M/1980/G4P10
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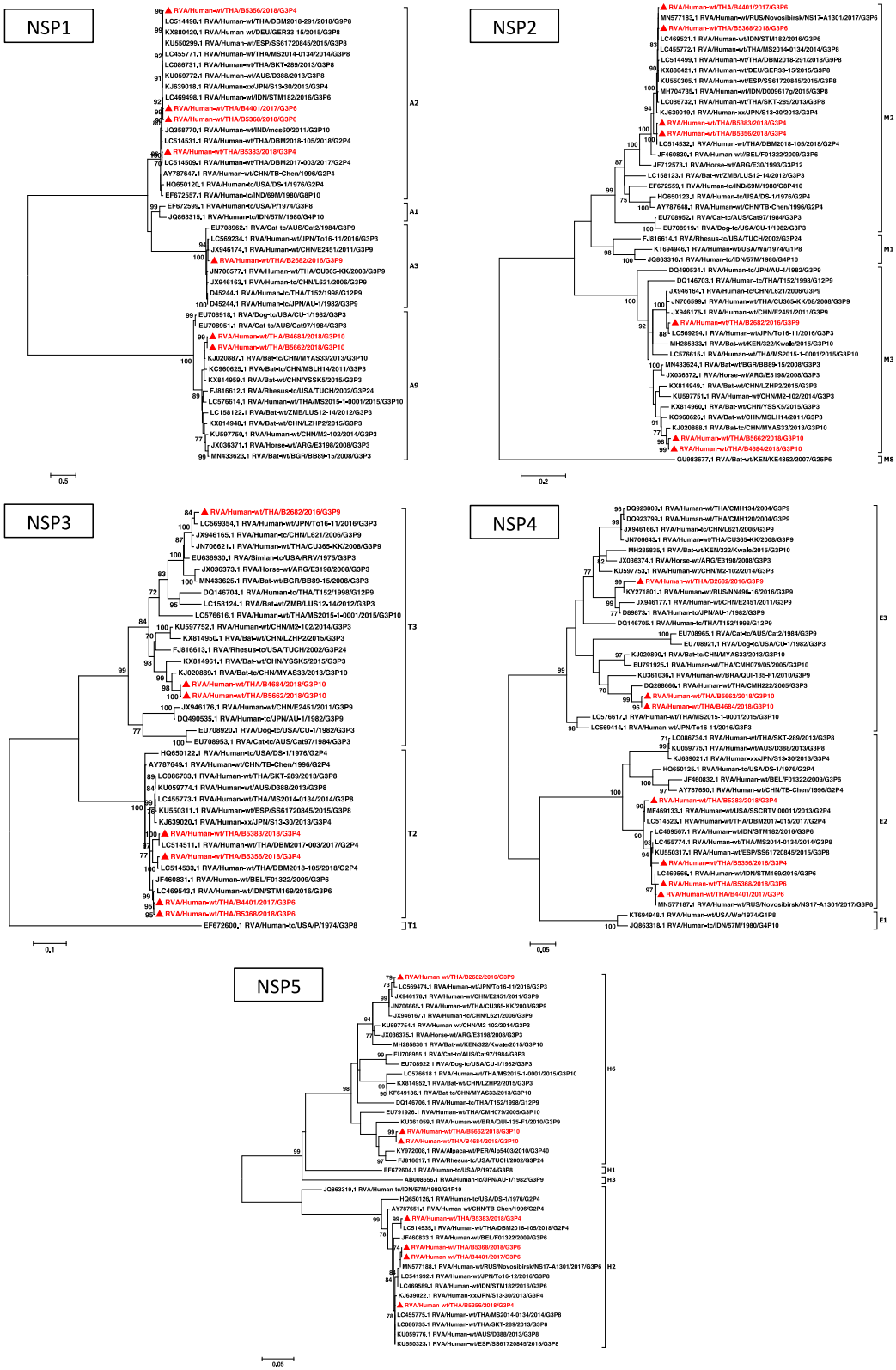
I1
0.1

Figure 8. **Phylogenetic analysis of the nucleotide sequences of G3 RVA structural protein genes.**

Thai strains (noted with triangles and in red) were compared to the reference and previously reported RVA strains. Nucleotide sequence lengths used in the maximum-likelihood phylogenetic analysis for VP1, VP2, VP3, VP4, VP6, and VP7 genes were 3204, 2528, 2507, 759, 1157, and 801 base pairs, respectively. Bootstrap values >70% are indicated at the tree nodes. Scale bars represent substitutions per nucleotide.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LC455771.1 RVA/Human-wt/THA/MS2014-0134/2014/G3P8
LC086731.1 RVA/Human-wt/THA/SKT-289/2013/G3P8
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KJ639018.1 RVA/Human-xx/JPN/S13-30/2013/G3P4
LC469498.1 RVA/Human-wt/IDN/STM182/2016/G3P6
RVA/Human-wt/THA/B4401/2017/G3P6
RVA/Human-wt/THA/B5368/2018/G3P6
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RVA/Human-wt/THA/B5383/2018/G3P4
LC514509.1 RVA/Human-wt/THA/DBM2017-003/2017/G2P4
AY787647.1 RVA/Human-wt/CHN/TB-Chen/1996/G2P4
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JQ863315.1 RVA/Human-tc/IDN/57M/1980/G4P10
EU708962.1 RVA/Cat-tc/AUS/Cat2/1984/G3P9
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EU708921.1 RVA/Dog-tc/USA/CU-1/1982/G3P3
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EU791925.1 RVA/Human-wt/THA/CMH079/05/2005/G3P10
KU361036.1 RVA/Human-wt/BRA/QUI-135-F1/2010/G3P9
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RVA/Human-wt/THA/B5356/2018/G3P4
LC469566.1 RVA/Human-wt/IDN/STM169/2016/G3P6
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FJ816617.1 RVA/Rhesus-tc/USA/TUCH/2002/G3P24
EF672604.1 RVA/Human-tc/USA/P/1974/G3P8
AB008656.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
JQ863319.1 RVA/Human-tc/IDN/57M/1980/G4P10
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LC514535.1 RVA/Human-wt/THA/DBM2018-105/2018/G2P4
JF460833.1 RVA/Human-wt/BEL/F01322/2009/G3P6
RVA/Human-wt/THA/B5368/2018/G3P6
RVA/Human-wt/THA/B4401/2017/G3P6
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KJ639022.1 RVA/Human-xx/JPN/S13-30/2013/G3P4
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H6
H1
H3
H2
0.05

Figure 9. **Phylogenetic analysis of the nucleotide sequences of G3 RVA non-structural protein genes.**

Thai strains (noted with triangles and in red) were compared to the reference and previously reported RVA strains. Nucleotide sequence lengths used in the maximum-likelihood phylogenetic analysis for NSP1, NSP2, NSP3, NSP4, and NSP5 genes were 1302, 953, 904, 618, and 520 base pairs, respectively. Bootstrap values >70% are indicated at the tree nodes. Scale bars represent substitutions per nucleotide.

Deduced amino acid residues of G3 strains mapped onto the VP7 trimer

VP7 has several defined antigenic epitopes (designated 7-1a, 7-1b, and 7-2). To determine amino acid changes between our G3 and the RotaTeq G3 vaccine strains and where these changes are located, we performed a sequence alignment. Although most antigenic residues were identical, notable differences were observed on residues 87 and 129 in epitope 7-1a, and residues 212, 213, 238, and 242 in epitope 7-1b (Fig 6). Specifically, G3P[4] possessed T87S and V129I (on epitope 7-1a) and N213T, K238D, and D242A (on epitope 7-1b). G3P[6] possessed all of the above changes except residue 129, which was identical to the vaccine strain. Moreover, G3P[9] differed from the vaccine strain only on epitope 7-1b (A212T, N213S, K238N, and D242N). Finally, G3P[10] was characterized by N213T, K238D, and D242T on epitope 7-1b.

A

	7-1a														7-1b						7-2									
	87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
RVA/Vaccine/USA/RotaTeq/WI78-8/1992/G3P7A5	T	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	A	N	K	D	K	D	A	T	L	S	E	A	G
RVA/Human-wt/THA/B5356/2018/G3P[4]	S	T	N	N	S	W	K	D	Q	D	A	I	D	K	Q	D	A	T	D	A	K	D	A	T	L	S	E	A	G
RVA/Human-wt/THA/B5383/2018/G3P[4]	S	T	N	N	S	W	K	D	Q	D	A	I	D	K	Q	D	A	T	D	A	K	D	A	T	L	S	E	A	G
RVA/Human-wt/THA/B4401/2017/G3P[6]	S	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	A	T	D	A	K	D	A	T	L	S	E	A	G
RVA/Human-wt/THA/B5368/2018/G3P[6]	S	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	A	T	D	A	K	D	A	T	L	S	E	A	G
RVA/Human-wt/THA/B2682/2016/G3P[9]	T	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	T	S	N	N	K	D	A	T	L	S	E	A	G
RVA/Human-wt/THA/B4684/2018/G3P[10]	T	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	A	T	D	T	K	D	A	T	L	S	E	A	G
RVA/Human-wt/THA/B5662/2018/G3P[10]	T	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	A	T	D	T	K	D	A	T	L	S	E	A	G

B

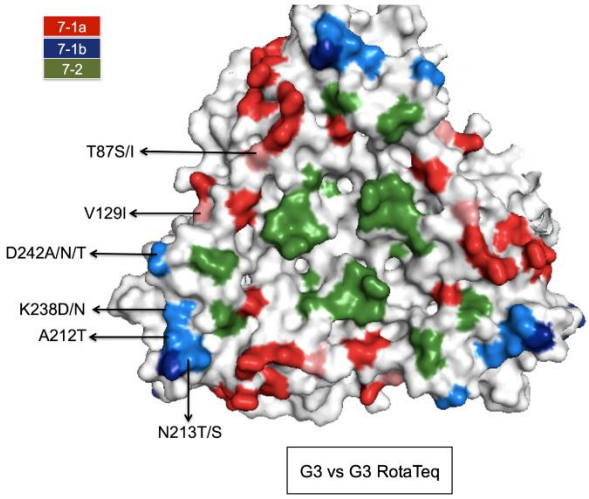

Figure 10. **Deduced VP7 amino acid residues of G3 strains mapped to the surface of the vaccine G3 VP7 trimer.**

(A) Alignment of the VP7 residues comprising the antigenic epitopes (7-1a, 71b, and 7-2). Residues different from RotaTeq are shaded light blue. (B) Surface representation of the VP7 trimer (PDB 3GZT). Antigenic epitopes are colored red (7-1a), blue (7-1b), and green (7-2). Surface-exposed residue differences between our G3 strains and G3 RotaTeq are lighter in color.

Discussion

RVA infection is traditionally associated with very young children, but occasional infection among adults do occur [36, 46]. In this study, we characterized the near-complete genome of seven atypical G3 strains, many of which were identified in adults who were unlikely to have been rotavirus-vaccinated. G3 strains represent diverse genome constellations with currently up to 20 RCWG-established genotypes [26]. The uncommon RVA strains from five infected adults versus two children may be attributed to adults' increased risk of infection with increasing age. Additional factors may also include occupational exposure, travel, or other life activities. Although patients from whom samples were derived live in and around Bangkok and likely did not have frequent contacts with wildlife, it is interesting to note that many of the gene segments closely resembled RVA of animal origins.

The G3P[4] and G3P[6] strains possessed equine-like G3, which was first detected in Japan in 2013 [228] and has since become predominant worldwide [123, 222, 238-242]. They possess similar genome constellation as the prototypic DS-1 except for the VP7 gene. From our analysis, they also exhibit high degree of identity (99-100%) with equine-like G3 from Russia (Novosibirsk/NS17-A1301) and Indonesia (STM182) [106].

Early detection of G3P[9] as represented by the prototypic AU-1 was from a patient in Japan in 1982 [243]. Human G3P[9] strains including our B2682 share common origins with feline RVA [235, 244, 245] and are infrequently detected in human [226, 246-250]. However, G3P[9] occasionally surfaced in Thailand (CMH120/04, CMH134/04, CU365) [233, 235]. The B2682 possessed identical genome constellation G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H6 as another Thai strain CU365 previously reported by our group [235] and strains L621 and E2451 from China [251]. It was observed that all G3P[9] strains regardless of host species possess an A3 NSP1 gene. This unique combination of P[9] and A3 was hypothesized to provide a replication advantage in various hosts [252].

Finding of G3P[10] in diarrheic adults living in Bangkok was unexpected. This genotype first appeared in 2005 during an epidemiological surveillance and was identified from a 2-year-old child hospitalized for severe diarrhea in northern Thailand [234]. Designated CMH079, this strain was not completely characterized at the time

and lacked genotype constellation information. Subsequent reports of G3P[10] were from a diarrheic 14-month-old child in eastern India (mcs60 strain) in 2011, and again in Thailand from an 11-month-old infant (MS2015-1-0001 strain) in 2015 [229, 253]. The most recently reported G3P[10] infection in Thailand was in a 1-year-old (CMH-S015-19) in January 2019 [254].

In our study, clinical symptoms reported by both adult patients with G3P[10] (B4684 and B5662) included fever, watery diarrhea, abdominal pain, vomiting, and dehydration. Although B4684 and B5662 were identified four months apart, their VP1 nucleotide sequences were sufficiently different to the genetically closest MYAS33 strain from bat (at 89% vs. 93%, respectively) that they are unlikely to be epidemiologically linked. Interestingly, the genome constellation of our G3P[10] and several previously described global strains differed by a single gene segment, either VP4 or VP6. For instance, our G3P[10] resembled the bat LZHP2, BB89-15, and BR89-60 strains, human M2-102 strain, and equine E3198 strain in all gene segments except VP4. Alternatively, B4684 and B5662 may be associated with another human strain MS2015-1-0001 first reported in northern Thailand. If so, the VP6 I3 in their genome constellation G3–P[10]–I3–R3–C3–M3–A9–N3–T3–E3–H6 had replaced the I8 in MS2015-1-0001 within the span of three years. It is noteworthy that the latter has an identical genotype constellation as a bat RVA strain MYAS33 identified in 2013 in China [255], which suggests probable zoonotic origin.

RotaTeq is a live attenuated vaccine containing reassortant strains of genotypes G1, G2, G3, G4, and P[8] [151]. It was initially feared that the rotavirus vaccines may impose selective pressure on circulating RVA strains, possibly influencing their evolutionary rate and the transmissibility of new RVA strains [34]. Moreover, the global emergence of equine-like G3 DS-1-like strains raised questions of whether vaccinations induced selective pressure on zoonotic RVA strains [256]. However, the use of rotavirus vaccines in Thailand is currently not widespread and Thai adults are extremely unlikely to have been vaccinated. When we mapped the deduced amino acid residue changes of our G3 strains onto the trimeric VP7 protein structure of the vaccine G3 strain, we identified several differences on the antigenic epitopes. Many of the differences were P type-specific, such as V129I in 7-1a for G3P[4] or D242T in 7-1b for G3P[10]. Residues of our G3P[9] differed most from the vaccine, particularly on the 7-1b

antigenic epitope. Interestingly, the K238N substitution, which is also present in various G3 strains from Argentina, Belgium, Pakistan, and Lebanon [224, 225, 257-259], can potentially introduce an N-linked glycosylation in the 7-1b epitope and reduce antibody neutralization [260].

This study has several limitations. We do not know whether there are unreported RVA infection of G3 genome constellation similar to ours elsewhere in Bangkok because RVA genome characterization is not typically done for viral diagnostics. Diarrheic adults do not always seek medical attention and only do so when symptoms are severe, therefore unreported cases may exist. We also do not know how our patients were infected as this information cannot be ascertained from the medical records at hand. It would be helpful to investigate whether any households of these patients had pets or other animals, which were also infected with RVA, to further examine animal-to-human transmission.

In conclusion, the G3 RVA identified in this study highlights unusual RVA genotype constellations even in urban settings, which would have otherwise eluded detection without a thorough genome analysis. Continued surveillance and molecular characterization of novel RVA in both human and animals are important to understand possible zoonosis and future strain inclusions in the vaccine.

CHAPTER VI

G3P[9] Rotavirus of Unusual Genome Constellation Identified in Diarrheic Cat in Thailand

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Abstract

Rotavirus infection can cause diarrhea in many animal species. A 2 year-old indoor female Siamese cat was ill with a mucus-bloody diarrhea and tested positive for rotavirus by real-time reverse-transcription polymerase chain reaction (RT-PCR). Subsequent conventional RT-PCR and nucleotide sequence analysis revealed a rotavirus G3P[9] genotype with the genome constellation G3-P[9]-I2-R2-C2-M2-A3-N2-T3-E3-H3. From phylogenetic analysis, the VP4, VP7, NSP1, NSP3, NSP4, and NSP5 genes were closely related to human/feline-like rotavirus, while VP1, VP2, VP3, VP6, and NSP2 genes were genetically closest to human bovine-like rotavirus. Although this G3P[9] strain was previously reported in Korea, which infected a 9 year-old girl (strain CAU-12-2-51) a decade ago, it has never been documented in Thailand and its emergence is enigmatic.

Keyword: Rotavirus, cat, feline, diarrhea, G3P[9], genome constellation

Introduction

Rotavirus (RV) causes diarrhea in humans and many animals species [66]. RV belongs to the family *Reoviridae* and possesses segmented double-stranded RNA genome. The virion is non-enveloped and has a triple-layered capsid [216]. RV genotyping relies on the two outer capsid proteins: VP7 is the glycoprotein (G) and VP4 is the protease-sensitive protein (P) [20]. Most recently, there are 41 G, 57 P, 31 I, 27 R, 23 C, 23 M, 38 A, 27 N, 27 T, 31 E, and 27 H genotypes among the RV identified in human and animal species worldwide [23].

The 11 RNA segments comprising the RV genome encode structural (VP1 to VP4, VP6, and VP7) and non-structural (NSP1 to NSP5) proteins. The segmented genome of RV is amenable to both intra- and inter-genogroup reassortment between and among strains [66], which can generate new genetic and antigenic variants [218]. The genotype constellations for RV reference strains have been designated by the Rotavirus Classification Working Group (RCWG), in which each of the 11 RV genome segments encodes VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 and corresponds to genotypes Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, respectively [25, 26]. The classification system provided important descriptive information on the genetic diversity of RV and often enabled the identification of reassortment and interspecies transmission events among human and animals [26].

Feline RV infection was first identified in cats in 1978 using serology [261]. Since feline RV are infrequent sources of zoonotic infections in humans [220, 262] and rarely caused severe illness in cats [263], it is not routinely screened in small animal veterinary practices in cases of diarrhea [264]. To date there are only three neutralizing antigen combinations that have been identified in

cats (G3P[3], G3P[9], and G6P[5]. Among these, 12 feline RV strains for which whole genome sequencing has been determined and six genotype constellations have been described to date [219, 220, 246, 265-267]. Potential interspecies transmission from cats to humans from RNA-RNA hybridization analysis data is consistent with findings that some feline RV (FRV1, FRV317, FRV381, and FRV384) are genetically related to human AU-1-like RV [262, 268]. In order to understand the interspecies transmission, reassortment, and evolutionary relationship between human and animal RV, full genome sequencing analysis is performed whenever possible [269]. To characterize a unique G3P[9] RV identified in a 2-year-old Siamese cat with diarrhea, we sequenced all 11 segments to determine its genome constellation.

Materials and Methods

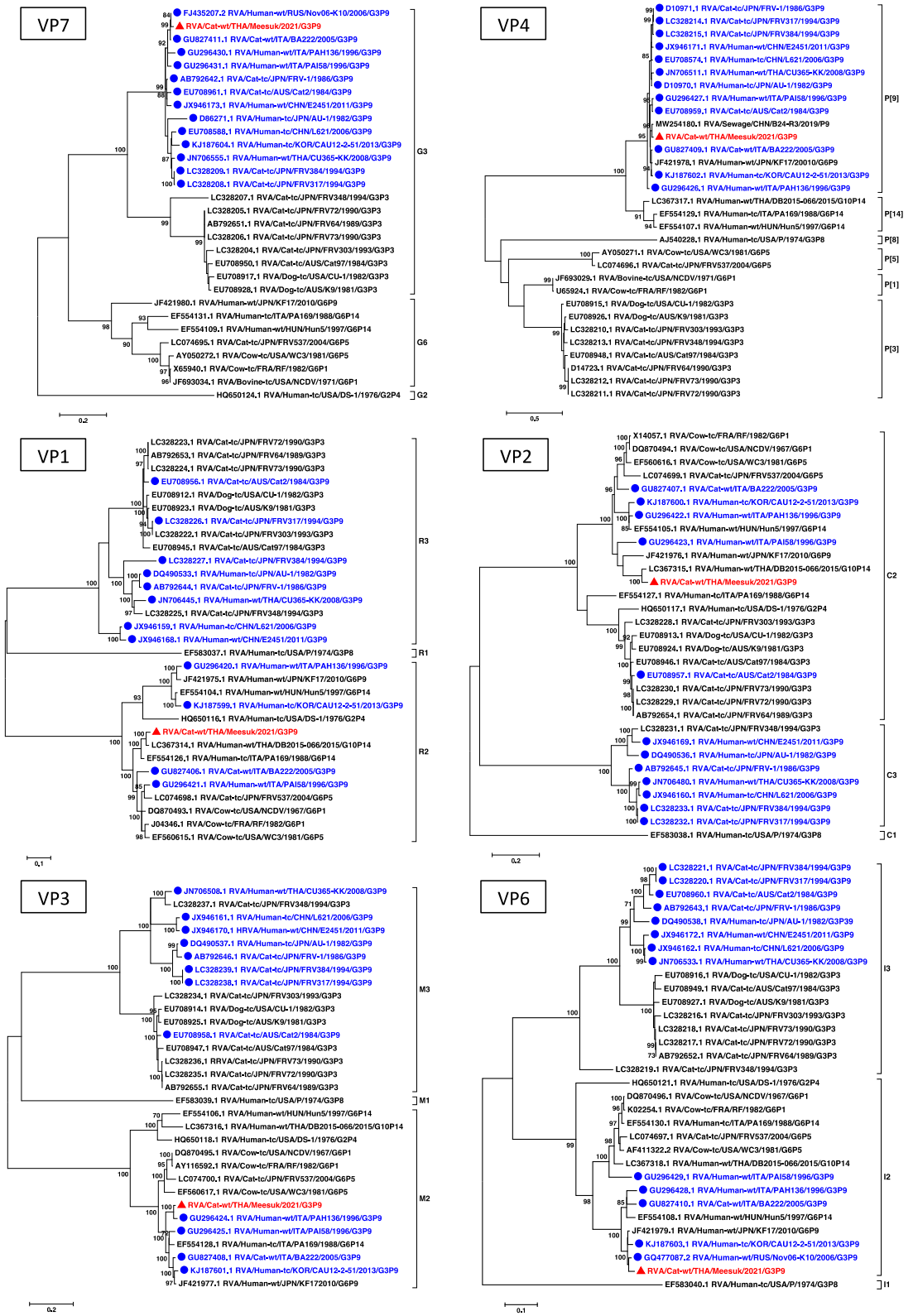
As part of the disease diagnostic procedure, residual stool sample from a cat with diarrhea previously tested negative for herpesvirus, parvovirus, enterovirus, and coronavirus was tested for RV. Stool suspension in phosphate-buffered saline was subjected to viral RNA extraction by using a magLEAD 12gC automated extraction system (Precision System Science, Chiba, Japan). One-step real-time reverse-transcription polymerase chain reaction (RT-PCR) to detect the partial NSP3 gene was performed with QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany) as previously described [203].

Conventional RT-PCR was performed with RV primers [204]. Briefly, the PCR conditions were 40 cycles of denaturation at 94 °C for 30 sec, annealing at 56 °C for 45 sec, and extension at 72 °C for 2 min. PCR product was treated with ExoSAP (GE Healthcare, USA) and Sanger sequenced. Nucleotide sequences were analyzed using BioEdit [237] and subjected to BLAST search (www.ncbi.nlm.nih.gov). Genotyping of all 11 gene segments was performed using the Rotavirus A Genotype Determination available through the Virus Pathogen Resource (ViPR) [75]. All sequences were deposited in the GenBank database under the accession numbers ON191596-ON191606.

Nucleotide sequences of individual gene segments were aligned with the RVA reference sequences using ClustalW. Maximum-likelihood phylogenetic trees were constructed with the best substitution models determined based on the corrected Bayesian information criterion value implemented in MEGA7 [208, 209]. Tree robustness was determined by bootstrapping of 1,000 replicates with values >70% considered significant.

Result

The nucleotide sequences of the VP7 and VP4 genes of this feline RV was consistent with the genotype G3P[9]. This strain was therefore designated RVA/Cat-wt/THA/Meesuk/2021/G3P[9] according to the standardized nomenclature. The nearly complete sequences for the remaining gene segments for structural (Fig. 11) and non-structural (Fig. 12) genes were analyzed by phylogenetic analyses and revealed the genome constellation G3–P[9]–I2–R2–C2–M2–A3–N2–T3–E3–H3. This constellation resembled the genotype of RV strain CAU12-2-51 identified in human in all gene segments and differed from the human RV strain KF17 only by the VP7 gene (Table 9). Nine out of 11 genotypes also resembled a feline strain BA222 (except NSP2 and NSP4) and the human RV strain PAI58 (except NSP3 and NSP4).


VP7
FJ435207.2 RVA/Human-wt/RUS/Nov06-K10/2006/G3P9
RVA/Cat-wt/THA/Meesuk/2021/G3P9
GU827411.1 RVA/Cat-wt/ITA/BA222/2005/G3P9
GU296430.1 RVA/Human-wt/ITA/PAH136/1996/G3P9
GU296431.1 RVA/Human-wt/ITA/PAI58/1996/G3P9
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JX946173.1 RVA/Human-wt/CHN/E2451/2011/G3P9
D86271.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
EU708588.1 RVA/Human-tc/CHN/L621/2006/G3P9
KJ187604.1 RVA/Human-tc/KOR/CAU12-2-51/2013/G3P9
JN706555.1 RVA/Human-wt/THA/CU365-KK/2008/G3P9
LC328209.1 RVA/Cat-tc/JPN/FRV384/1994/G3P9
LC328208.1 RVA/Cat-tc/JPN/FRV317/1994/G3P9
LC328207.1 RVA/Cat-tc/JPN/FRV348/1994/G3P3
LC328205.1 RVA/Cat-tc/JPN/FRV72/1990/G3P3
AB792651.1 RVA/Cat-tc/JPN/FRV64/1989/G3P3
LC328206.1 RVA/Cat-tc/JPN/FRV73/1990/G3P3
LC328204.1 RVA/Cat-tc/JPN/FRV303/1993/G3P3
EU708950.1 RVA/Cat-tc/AUS/Cat97/1984/G3P3
EU708917.1 RVA/Dog-tc/USA/CU-1/1982/G3P3
EU708928.1 RVA/Dog-tc/AUS/K9/1981/G3P3
JF421980.1 RVA/Human-wt/JPN/KF17/2010/G6P9
EF554131.1 RVA/Human-tc/ITA/PA169/1988/G6P14
EF554109.1 RVA/Human-wt/HUN/Hun5/1997/G6P14
LC074695.1 RVA/Cat-tc/JPN/FRV537/2004/G6P5
AY050272.1 RVA/Cow-tc/USA/WC3/1981/G6P5
X65940.1 RVA/Cow-tc/FRA/RF/1982/G6P1
JF693034.1 RVA/Bovine-tc/USA/NCDV/1971/G6P1
HQ650124.1 RVA/Human-tc/USA/DS-1/1976/G2P4
G3
G6
G2
0.2
VP4
D10971.1 RVA/Cat-tc/JPN/FRV-1/1986/G3P9
LC328214.1 RVA/Cat-tc/JPN/FRV317/1994/G3P9
LC328215.1 RVA/Cat-tc/JPN/FRV384/1994/G3P9
JX946171.1 RVA/Human-wt/CHN/E2451/2011/G3P9
EU708574.1 RVA/Human-tc/CHN/L621/2006/G3P9
JN706511.1 RVA/Human-wt/THA/CU365-KK/2008/G3P9
D10970.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
GU296427.1 RVA/Human-wt/ITA/PAI58/1996/G3P9
EU708959.1 RVA/Cat-tc/AUS/Cat2/1984/G3P9
MW254180.1 RVA/Sewage/CHN/B24-R3/2019/P9
RVA/Cat-wt/THA/Meesuk/2021/G3P9
GU827409.1 RVA/Cat-wt/ITA/BA222/2005/G3P9
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KJ187602.1 RVA/Human-tc/KOR/CAU12-2-51/2013/G3P9
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EF554107.1 RVA/Human-wt/HUN/Hun5/1997/G6P14
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AY050271.1 RVA/Cow-tc/USA/WC3/1981/G6P5
LC074696.1 RVA/Cat-tc/JPN/FRV537/2004/G6P5
JF693029.1 RVA/Bovine-tc/USA/NCDV/1971/G6P1
U65924.1 RVA/Cow-tc/FRA/RF/1982/G6P1
EU708915.1 RVA/Dog-tc/USA/CU-1/1982/G3P3
EU708926.1 RVA/Dog-tc/AUS/K9/1981/G3P3
LC328210.1 RVA/Cat-tc/JPN/FRV303/1993/G3P3
LC328213.1 RVA/Cat-tc/JPN/FRV348/1994/G3P3
EU708948.1 RVA/Cat-tc/AUS/Cat97/1984/G3P3
D14723.1 RVA/Cat-tc/JPN/FRV64/1990/G3P3
LC328212.1 RVA/Cat-tc/JPN/FRV73/1990/G3P3
LC328211.1 RVA/Cat-tc/JPN/FRV72/1990/G3P3
P[9]
P[14]
P[8]
P[5]
P[1]
P[3]
0.5
VP1
LC328223.1 RVA/Cat-tc/JPN/FRV72/1990/G3P3
AB792653.1 RVA/Cat-tc/JPN/FRV64/1989/G3P3
LC328224.1 RVA/Cat-tc/JPN/FRV73/1990/G3P3
EU708956.1 RVA/Cat-tc/AUS/Cat2/1984/G3P9
EU708912.1 RVA/Dog-tc/USA/CU-1/1982/G3P3
EU708923.1 RVA/Dog-tc/AUS/K9/1981/G3P3
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LC328222.1 RVA/Cat-tc/JPN/FRV303/1993/G3P3
EU708945.1 RVA/Cat-tc/AUS/Cat97/1984/G3P3
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DQ490533.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
AB792644.1 RVA/Cat-tc/JPN/FRV-1/1986/G3P9
JN706445.1 RVA/Human-wt/THA/CU365-KK/2008/G3P9
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EF554104.1 RVA/Human-wt/HUN/Hun5/1997/G6P14
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J04346.1 RVA/Cow-tc/FRA/RF/1982/G6P1
EF560615.1 RVA/Cow-tc/USA/WC3/1981/G6P5
R3
R1
R2
0.1
VP2
X14057.1 RVA/Cow-tc/FRA/RF/1982/G6P1
DQ870494.1 RVA/Cow-tc/USA/NCDV/1967/G6P1
EF560616.1 RVA/Cow-tc/USA/WC3/1981/G6P5
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GU296423.1 RVA/Human-wt/ITA/PAI58/1996/G3P9
JF421976.1 RVA/Human-wt/JPN/KF17/2010/G6P9
LC367315.1 RVA/Human-wt/THA/DB2015-066/2015/G10P14
RVA/Cat-wt/THA/Meesuk/2021/G3P9
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HQ650117.1 RVA/Human-tc/USA/DS-1/1976/G2P4
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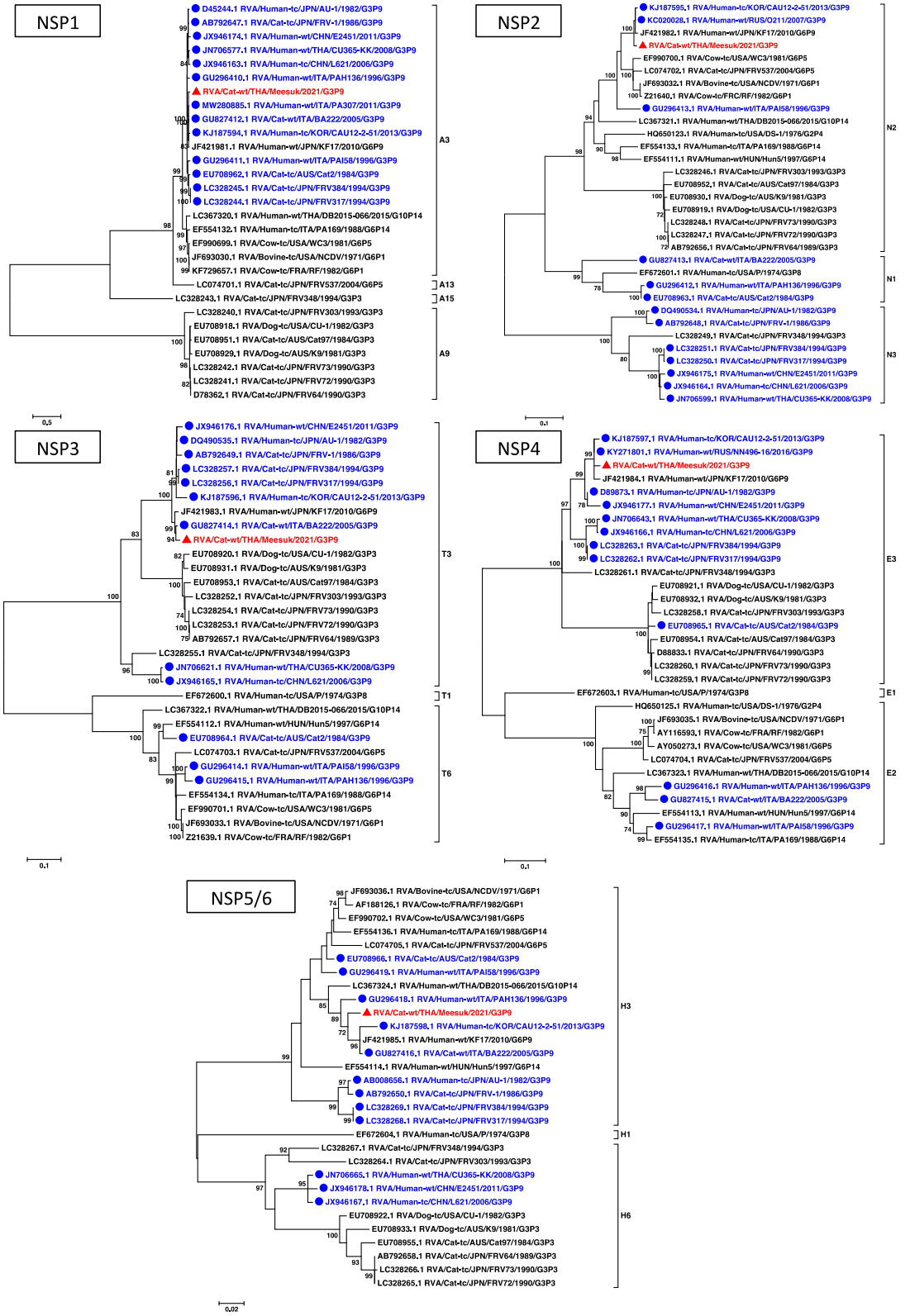
EU708913.1 RVA/Dog-tc/USA/CU-1/1982/G3P3
EU708924.1 RVA/Dog-tc/AUS/K9/1981/G3P3
EU708946.1 RVA/Cat-tc/AUS/Cat97/1984/G3P3
EU708957.1 RVA/Cat-tc/AUS/Cat2/1984/G3P9
LC328230.1 RVA/Cat-tc/JPN/FRV73/1990/G3P3
LC328229.1 RVA/Cat-tc/JPN/FRV72/1990/G3P3
AB792654.1 RVA/Cat-tc/JPN/FRV64/1989/G3P3
LC328231.1 RVA/Cat-tc/JPN/FRV348/1994/G3P3
JX946169.1 RVA/Human-wt/CHN/E2451/2011/G3P9
DQ490536.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
AB792645.1 RVA/Cat-tc/JPN/FRV-1/1986/G3P9
JN706480.1 RVA/Human-wt/THA/CU365-KK/2008/G3P9
JX946160.1 RVA/Human-tc/CHN/L621/2006/G3P9
LC328233.1 RVA/Cat-tc/JPN/FRV384/1994/G3P9
LC328232.1 RVA/Cat-tc/JPN/FRV317/1994/G3P9
EF583038.1 RVA/Human-tc/USA/P/1974/G3P8
C2
C3
C1
0.2
VP3
JN706508.1 RVA/Human-wt/THA/CU365-KK/2008/G3P9
LC328237.1 RVA/Cat-tc/JPN/FRV348/1994/G3P3
JX946161.1 RVA/Human-tc/CHN/L621/2006/G3P9
JX946170.1 HRVA/Human-wt/CHN/E2451/2011/G3P9
DQ490537.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
AB792646.1 RVA/Cat-tc/JPN/FRV-1/1986/G3P9
LC328239.1 RVA/Cat-tc/JPN/FRV384/1994/G3P9
LC328238.1 RVA/Cat-tc/JPN/FRV317/1994/G3P9
LC328234.1 RVA/Cat-tc/JPN/FRV303/1993/G3P3
EU708914.1 RVA/Dog-tc/USA/CU-1/1982/G3P3
EU708925.1 RVA/Dog-tc/AUS/K9/1981/G3P3
EU708958.1 RVA/Cat-tc/AUS/Cat2/1984/G3P9
EU708947.1 RVA/Cat-tc/AUS/Cat97/1984/G3P3
LC328236.1 RVA/Cat-tc/JPN/FRV73/1990/G3P3
LC328235.1 RVA/Cat-tc/JPN/FRV72/1990/G3P3
AB792655.1 RVA/Cat-tc/JPN/FRV64/1989/G3P3
EF583039.1 RVA/Human-tc/USA/P/1974/G3P8
EF554106.1 RVA/Human-wt/HUN/Hun5/1997/G6P14
LC367316.1 RVA/Human-wt/THA/DB2015-066/2015/G10P14
HQ650118.1 RVA/Human-tc/USA/DS-1/1976/G2P4
DQ870495.1 RVA/Cow-tc/USA/NCDV/1967/G6P1
AY116592.1 RVA/Cow-tc/FRA/RF/1982/G6P1
LC074700.1 RVA/Cat-tc/JPN/FRV537/2004/G6P5
EF560617.1 RVA/Cow-tc/USA/WC3/1981/G6P5
RVA/Cat-wt/THA/Meesuk/2021/G3P9
GU296424.1 RVA/Human-wt/ITA/PAH136/1996/G3P9
GU296425.1 RVA/Human-wt/ITA/PAI58/1996/G3P9
EF554128.1 RVA/Human-tc/ITA/PA169/1988/G6P14
GU827408.1 RVA/Cat-wt/ITA/BA222/2005/G3P9
KJ187601.1 RVA/Human-tc/KOR/CAU12-2-51/2013/G3P9
JF421977.1 RVA/Human-wt/JPN/KF172010/G6P9
M3
M1
M2
0.2
VP6
LC328221.1 RVA/Cat-tc/JPN/FRV384/1994/G3P9
LC328220.1 RVA/Cat-tc/JPN/FRV317/1994/G3P9
EU708960.1 RVA/Cat-tc/AUS/Cat2/1984/G3P9
AB792643.1 RVA/Cat-tc/JPN/FRV-1/1986/G3P9
DQ490538.1 RVA/Human-tc/JPN/AU-1/1982/G3P39
JX946172.1 RVA/Human-wt/CHN/E2451/2011/G3P9
JX946162.1 RVA/Human-tc/CHN/L621/2006/G3P9
JN706533.1 RVA/Human-wt/THA/CU365-KK/2008/G3P9
EU708916.1 RVA/Dog-tc/USA/CU-1/1982/G3P3
EU708949.1 RVA/Cat-tc/AUS/Cat97/1984/G3P3
EU708927.1 RVA/Dog-tc/AUS/K9/1981/G3P3
LC328216.1 RVA/Cat-tc/JPN/FRV303/1993/G3P3
LC328218.1 RVA/Cat-tc/JPN/FRV73/1990/G3P3
LC328217.1 RVA/Cat-tc/JPN/FRV72/1990/G3P3
AB792652.1 RVA/Cat-tc/JPN/FRV64/1989/G3P3
LC328219.1 RVA/Cat-tc/JPN/FRV348/1994/G3P3
HQ650121.1 RVA/Human-tc/USA/DS-1/1976/G2P4
DQ870496.1 RVA/Cow-tc/USA/NCDV/1967/G6P1
K02254.1 RVA/Cow-tc/FRA/RF/1982/G6P1
EF554130.1 RVA/Human-tc/ITA/PA169/1988/G6P14
LC074697.1 RVA/Cat-tc/JPN/FRV537/2004/G6P5
AF411322.2 RVA/Cow-tc/USA/WC3/1981/G6P5
LC367318.1 RVA/Human-wt/THA/DB2015-066/2015/G10P14
GU296429.1 RVA/Human-wt/ITA/PAI58/1996/G3P9
GU296428.1 RVA/Human-wt/ITA/PAH136/1996/G3P9
GU827410.1 RVA/Cat-wt/ITA/BA222/2005/G3P9
EF554108.1 RVA/Human-wt/HUN/Hun5/1997/G6P14
JF421979.1 RVA/Human-wt/JPN/KF17/2010/G6P9
KJ187603.1 RVA/Human-tc/KOR/CAU12-2-51/2013/G3P9
GQ477087.2 RVA/Human-wt/RUS/Nov06-K10/2006/G3P9
RVA/Cat-wt/THA/Meesuk/2021/G3P9
EF583040.1 RVA/Human-tc/USA/P/1974/G3P8
I3
I2
I1
0.1

Figure 11. **Phylogenetic analysis of the nucleotide sequences of the RV structural protein genes.**

Feline RV from this study (red and triangled) was compared to other G3P[9] (blue and dotted) and global strains (black). Nucleotide sequence lengths used in the maximum-likelihood phylogenetic analysis for VP1, VP2, VP3, VP4, VP6, and VP7 genes were 3253, 2518, 2465, 659, 1194, and 980 base pairs, respectively. Bootstrap values >70% are indicated at the tree nodes. Scale bars represent substitutions per nucleotide.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JN706577.1 RVA/Human-wt/THA/CU365-KK/2008/G3P9
JX946163.1 RVA/Human-tc/CHN/L621/2006/G3P9
GU296410.1 RVA/Human-wt/ITA/PAH136/1996/G3P9
RVA/Cat-wt/THA/Meesuk/2021/G3P9
MW280885.1 RVA/Human-wt/ITA/PA307/2011/G3P9
GU827412.1 RVA/Cat-wt/ITA/BA222/2005/G3P9
KJ187594.1 RVA/Human-tc/KOR/CAU12-2-51/2013/G3P9
JF421981.1 RVA/Human-wt/JPN/KF17/2010/G6P9
GU296411.1 RVA/Human-wt/ITA/PAI58/1996/G3P9
EU708962.1 RVA/Cat-tc/AUS/Cat2/1984/G3P9
LC328245.1 RVA/Cat-tc/JPN/FRV384/1994/G3P9
LC328244.1 RVA/Cat-tc/JPN/FRV317/1994/G3P9
LC367320.1 RVA/Human-wt/THA/DB2015-066/2015/G10P14
EF554132.1 RVA/Human-tc/ITA/PA169/1988/G6P14
EF990699.1 RVA/Cow-tc/USA/WC3/1981/G6P5
JF693030.1 RVA/Bovine-tc/USA/NCDV/1971/G6P1
KF729657.1 RVA/Cow-tc/FRA/RF/1982/G6P1
LC074701.1 RVA/Cat-tc/JPN/FRV537/2004/G6P5
LC328243.1 RVA/Cat-tc/JPN/FRV348/1994/G3P3
LC328240.1 RVA/Cat-tc/JPN/FRV303/1993/G3P3
EU708918.1 RVA/Dog-tc/USA/CU-1/1982/G3P3
EU708951.1 RVA/Cat-tc/AUS/Cat97/1984/G3P3
EU708929.1 RVA/Dog-tc/AUS/K9/1981/G3P3
LC328242.1 RVA/Cat-tc/JPN/FRV73/1990/G3P3
LC328241.1 RVA/Cat-tc/JPN/FRV72/1990/G3P3
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A3
A13
A15
A9
0.5
NSP2
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RVA/Cat-wt/THA/Meesuk/2021/G3P9
EF990700.1 RVA/Cow-tc/USA/WC3/1981/G6P5
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Z21640.1 RVA/Cow-tc/FRC/RF/1982/G6P1
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EF554111.1 RVA/Human-wt/HUN/Hun5/1997/G6P14
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EU708930.1 RVA/Dog-tc/AUS/K9/1981/G3P3
EU708919.1 RVA/Dog-tc/USA/CU-1/1982/G3P3
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LC328247.1 RVA/Cat-tc/JPN/FRV72/1990/G3P3
AB792656.1 RVA/Cat-tc/JPN/FRV64/1989/G3P3
GU827413.1 RVA/Cat-wt/ITA/BA222/2005/G3P9
EF672601.1 RVA/Human-tc/USA/P/1974/G3P8
GU296412.1 RVA/Human-wt/ITA/PAH136/1996/G3P9
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JX946164.1 RVA/Human-tc/CHN/L621/2006/G3P9
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N2
N1
N3
0.1
NSP3
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DQ490535.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
AB792649.1 RVA/Cat-tc/JPN/FRV-1/1986/G3P9
LC328257.1 RVA/Cat-tc/JPN/FRV384/1994/G3P9
LC328256.1 RVA/Cat-tc/JPN/FRV317/1994/G3P9
KJ187596.1 RVA/Human-tc/KOR/CAU12-2-51/2013/G3P9
JF421983.1 RVA/Human-wt/JPN/KF17/2010/G6P9
GU827414.1 RVA/Cat-wt/ITA/BA222/2005/G3P9
RVA/Cat-wt/THA/Meesuk/2021/G3P9
EU708920.1 RVA/Dog-tc/USA/CU-1/1982/G3P3
EU708931.1 RVA/Dog-tc/AUS/K9/1981/G3P3
EU708953.1 RVA/Cat-tc/AUS/Cat97/1984/G3P3
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LC328253.1 RVA/Cat-tc/JPN/FRV72/1990/G3P3
AB792657.1 RVA/Cat-tc/JPN/FRV64/1989/G3P3
LC328255.1 RVA/Cat-tc/JPN/FRV348/1994/G3P3
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JX946165.1 RVA/Human-tc/CHN/L621/2006/G3P9
EF672600.1 RVA/Human-tc/USA/P/1974/G3P8
LC367322.1 RVA/Human-wt/THA/DB2015-066/2015/G10P14
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LC074703.1 RVA/Cat-tc/JPN/FRV537/2004/G6P5
GU296414.1 RVA/Human-wt/ITA/PAI58/1996/G3P9
GU296415.1 RVA/Human-wt/ITA/PAH136/1996/G3P9
EF554134.1 RVA/Human-tc/ITA/PA169/1988/G6P14
EF990701.1 RVA/Cow-tc/USA/WC3/1981/G6P5
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Z21639.1 RVA/Cow-tc/FRA/RF/1982/G6P1
T3
T1
T6
0.1
NSP4
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RVA/Cat-wt/THA/Meesuk/2021/G3P9
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EU708965.1 RVA/Cat-tc/AUS/Cat2/1984/G3P9
EU708954.1 RVA/Cat-tc/AUS/Cat97/1984/G3P3
D88833.1 RVA/Cat-tc/JPN/FRV64/1990/G3P3
LC328260.1 RVA/Cat-tc/JPN/FRV73/1990/G3P3
LC328259.1 RVA/Cat-tc/JPN/FRV72/1990/G3P3
EF672603.1 RVA/Human-tc/USA/P/1974/G3P8
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AY050273.1 RVA/Cow-tc/USA/WC3/1981/G6P5
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GU827415.1 RVA/Cat-wt/ITA/BA222/2005/G3P9
EF554113.1 RVA/Human-wt/HUN/Hun5/1997/G6P14
GU296417.1 RVA/Human-wt/ITA/PAI58/1996/G3P9
EF554135.1 RVA/Human-tc/ITA/PA169/1988/G6P14
E3
E1
E2
0.1
NSP5/6
JF693036.1 RVA/Bovine-tc/USA/NCDV/1971/G6P1
AF188126.1 RVA/Cow-tc/FRA/RF/1982/G6P1
EF990702.1 RVA/Cow-tc/USA/WC3/1981/G6P5
EF554136.1 RVA/Human-tc/ITA/PA169/1988/G6P14
LC074705.1 RVA/Cat-tc/JPN/FRV537/2004/G6P5
EU708966.1 RVA/Cat-tc/AUS/Cat2/1984/G3P9
GU296419.1 RVA/Human-wt/ITA/PAI58/1996/G3P9
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GU296418.1 RVA/Human-wt/ITA/PAH136/1996/G3P9
RVA/Cat-wt/THA/Meesuk/2021/G3P9
KJ187598.1 RVA/Human-tc/KOR/CAU12-2-51/2013/G3P9
JF421985.1 RVA/Human-wt/KF17/2010/G6P9
GU827416.1 RVA/Cat-wt/ITA/BA222/2005/G3P9
EF554114.1 RVA/Human-wt/HUN/Hun5/1997/G6P14
AB008656.1 RVA/Human-tc/JPN/AU-1/1982/G3P9
AB792650.1 RVA/Cat-tc/JPN/FRV-1/1986/G3P9
LC328269.1 RVA/Cat-tc/JPN/FRV384/1994/G3P9
LC328268.1 RVA/Cat-tc/JPN/FRV317/1994/G3P9
EF672604.1 RVA/Human-tc/USA/P/1974/G3P8
LC328267.1 RVA/Cat-tc/JPN/FRV348/1994/G3P3
LC328264.1 RVA/Cat-tc/JPN/FRV303/1993/G3P3
JN706665.1 RVA/Human-wt/THA/CU365-KK/2008/G3P9
JX946178.1 RVA/Human-wt/CHN/E2451/2011/G3P9
JX946167.1 RVA/Human-tc/CHN/L621/2006/G3P9
EU708922.1 RVA/Dog-tc/USA/CU-1/1982/G3P3
EU708933.1 RVA/Dog-tc/AUS/K9/1981/G3P3
EU708955.1 RVA/Cat-tc/AUS/Cat97/1984/G3P3
AB792658.1 RVA/Cat-tc/JPN/FRV64/1989/G3P3
LC328266.1 RVA/Cat-tc/JPN/FRV73/1990/G3P3
LC328265.1 RVA/Cat-tc/JPN/FRV72/1990/G3P3
H3
H1
H6
0.02

Figure 12. **Phylogenetic trees of the nucleotide sequences of the RV non-structural protein genes.**

Feline RV from this study (red and triangled) was compared to other G3P[9] (blue and dotted) and global strains (black). Nucleotide sequence lengths used in the maximum-likelihood phylogenetic analysis for NSP1, NSP2, NSP3, NSP4, and NSP5 genes were 1455, 950, 915, 528, and 591 base pairs, respectively. Bootstrap values >70% are indicated at the tree nodes. Scale bars represent substitutions per nucleotide.

Table 9. **Comparison of the feline RV genome constellation in this study to other human and non-human strains.**

Strain	Host	Genotype										
		VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5/6
RVA/Cat-wt/THA/Meesuk/2021/G3P[9]	**Feline**	**G3**	**P[9]**	**I2**	**R2**	**C2**	**M2**	**A3**	**N2**	**T3**	**E3**	**H3**
RVA/Human-tc/KOR/CAU12-2-51/2013/G3P[9]	**Human**	**G3**	**P[9]**	**I2**	**R2**	**C2**	**M2**	**A3**	**N2**	**T3**	**E3**	**H3**
RVA/Human-wt/JPN/KF17/2010/G6P[9]	Human	G6	P[9]	I2	R2	C2	M2	A3	N2	T3	E3	H3
RVA/Cat-wt/ITA/BA222/2005/G3P[9]	Feline	G3	P[9]	I2	R2	C2	M2	A3	N1	T3	E2	H3
RVA/Human-wt/ITA/PAI58/1996/G3P[9]	Human	G3	P[9]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Human-wt/ITA/PAH136/1996/G3P[9]	Human	G3	P[9]	I2	R2	C2	M2	A3	N1	T6	E2	H3
RVA/Human-tc/ITA/PA169/1988/G6P[14]	Human	G6	P[14]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Cow-tc/USA/WC3/1981/G6P[5]	Bovine	G6	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Cow-tc/FRA/RF/1982/G6P[1]	Bovine	G6	P[1]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Cat-tc/AUS/Cat2/1984/G3P[9]	Feline	G3	P[9]	I3	R3	C2	M3	A3	N1	T6	E3	H3
RVA/Cat-tc/JPN/FRV-1/1986/G3P[9]	Feline	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Cat-tc/JPN/FRV317/1994/G3P[9]	Feline	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Cat-tc/JPN/FRV384/1994/G3P[9]	Feline	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Human-wt/JPN/AU-1/1982/G3P[9]	Human	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Dog-wt/THA/CU23379/2019/G3P[3]	Canine	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6

The nucleotide sequences of the VP1 and VP2 genes of our feline RV were most closely related to the human bovine-like DB2015-066 strain (97.5% and 96.4%, respectively) (Table 10) and thus clustered together on the phylogenetic trees (Fig.11). VP3 gene was closely related (~98% nucleotide identity) to the Italian human feline-like PAH136. Interestingly, the VP4 gene of our strain shared highest nucleotide identity (~98% identity) with RV strain collected from sewage in China (B24-R3). Both VP6 and VP7 genes exhibit the highest nucleotide identity with the Russian RV strain Nov06-K10.

Among the non-structural genes, the NSP1 demonstrated the highest degree of sequence identity to the Italian strain PA307 (~97%) and all of G3P[9] strains clustered together in the A3 genotype (Fig. 12). The NSP2 and NSP4 gene exhibited ~99% nucleotide identity with Russian strain O211 and NN496-16, respectively. Meanwhile, the NPS3 gene sequences resembled the feline RV strain BA222 (~99% identity). The NSP5 gene shared ~98% identity with Japanese strain KF17, which consistently clustered with all non-structural genes from our feline RV on the phylogenetic trees.

Taken together, the feline RV described in this study was identical in the genome constellation to the CAU12-2-51 strain described in a human infection 10 years ago, but was distinctively different from the closest feline RV strain described in the literature.

Table 10. **Nucleotide sequence identities of all 11 segments of the feline RV compared to the genetically closest related RV strains.**

Gene	Cut off value*	Genotype	Strain showing highest identity	Percentage (%)	Percentage (%) identity with other strain						
					CAU12-2-51	KF17	BA222	PAI58	PAH136	AU-1	WC3
VP7	80	G3	RVA/Human-wt/RUS/Nov06-K10/2006/G3P[9]	98.93	93.49	G6	98.64	96.31	95.14	92.81	G6
VP4	80	P[9]	RVA/Sewage/CHN/B24-R3/2019/P[9]	97.59	95.93	95.93	96.08	94.87	94.12	95.17	P[1]
VP6	85	I2	RVA/Human-wt/RUS/Nov06-K10/2006/G3P[9]	98.39	96.87	97.55	93.96	87.66	94.11	I3	88.36
VP1	83	R2	RVA/Human-wt/THA/DB2015-066/2015/G10P[14]	97.52	85.82	85.15	91.14	90.26	85.03	R3	90.92
VP2	84	C2	RVA/Human-wt/THA/DB2015-066/2015/G10P[14]	96.39	88.08	88.72	88.90	88.83	88.42	C3	88.50
VP3	81	M2	RVA/Human-wt/ITA/PAH136/1996/G3P[9]	97.73	92.56	92.91	93.11	93.54	97.73	M3	89.66
NSP1	79	A3	RVA/Human-wt/ITA/PA307/2011/G3P[9]	97.26	95.85	96.72	97.05	92.17	93.04	93.37	84.01
NSP2	85	N2	RVA/Human-wt/RUS/O211/2007/G3P[9]	99.01	98.42	98.41	N1	92.42	N1	N3	93.56
NSP3	85	T3	RVA/Cat-wt/ITA/BA222/2005/G3P[9]	98.55	94.78	97.78	98.55	T6	T6	96.62	T6
NSP4	85	E3	RVA/Human-wt/RUS/NN496-16/2016/G3P[9]	98.99	98.13	97.99	E2	E2	E2	96.83	E2
NSP5	91	H3	RVA/Human-wt/JPN/KF17/2010/G6P[9]	98.39	97.27	98.39	98.23	95.66	97.75	93.58	95.35

***** Percentage nucleotide cut-off values and genotype proposed by Matthijnssens et al., 2008.

Discussion

There are few reports of feline RV when compared to the studies of RV infection in human and other animal species. Our characterization of another RV genome constellation identified from a diarrheic cat adds to the existing knowledge of a feline RV with a defined genotype. Most feline RV strains in the existing database have similar genome constellation as the AU-1 strain (in which most segments are of genotype 3) with a few exceptions. Historically, the G3P[3] and G3P[9] strains are particularly common feline RV [267]. Although the G3P[3] strains often possess highly conserved genome constellation of G3-P[3]-I3-R3-C2-M3-A9-N2-T3-E3-H6 [270], the G3P[9] strains appear to be more diverse [249]. The feline RV genome constellation identified in our study (G3–P[9]–I2–R2–C2–M2–A3–N2–T3–E3–H3) was genetically closest to the feline BA222 strain, but still differed by two gene segments [246]. Our strain has N2 instead of N1 for NSP2, and E3 instead of E2 for NSP4. Interestingly, RV with identical genome constellation described in our study was previously isolated from an unvaccinated 9-year-old Korean girl who presented with severe gastroenteritis (CAU12-2-51 strain) in 2012 [244]. That patient reportedly not had any contact with animals prior to hospitalization. Our laboratory routine performs molecular surveillance of RV in hospitalized patients, but we have not detected any infection with this feline RV genome constellation within the past 10 years. For comparison, the next most similar RV was reported in Japan from a 3-year-old girl hospitalized with acute gastroenteritis (KF17 strain). Although that strain was a G6P[9], the genome constellation was otherwise identical to ours [269]. In the latter study, the authors eluded to the fact that the combination T3 genotype in the NSP3 gene, E3 genotype in the NSP4 gene,

and H3 in the NSP5 gene are unique to the prototypic AU-1 human RV, which the feline RV strain BA222 also possessed.

Previous report concluded that the genome constellation of the CAU12-2-51 strain suggests a complex evolutionary origin, potentially involving reassortment events among feline, human, and bovine RV. The genes encoding VP4, VP7, NSP1, NSP3, NSP4, NSP5 were related to human feline AU-1-like, while the remaining genes were similar to the human bovine DS1-like and bovine RV strains. Except for the VP7 gene, KF-17 strain was also similar [244, 269]. It has been hypothesized that the human RV strains belonging to the AU-1-like and DS-1-like genotypes had a close evolutionary relationship with feline, canine, and bovine RV strains [220].

Whether this feline RV strain emerged in Thailand by chance alone or as a result of transboundary transmission is unknown. Two gene segments, VP1 and VP2, were genetically closest to the DB2015-066 strain, which coincidentally was identified in a one-year-old Thai girl with severe diarrhea in 2015. The remaining gene segments were more closely related to other global strains than any RV strains previously reported in Thailand. The observed co-existence of the genotype combination N2 of NSP2 gene and E3 of NSP4 gene have previously been reported in Thailand in 2020 in canine RV infection in 2-month-old healthy mixed breed and diarrheic beagle and German shepherd puppies [271]. However, our feline RV NSP2 gene only shared ~95% nucleotide identity with those canine RV strains, while exhibited >99% nucleotide identity to the human-bovine-like strain O211. Our feline RV NSP4 gene shared ~91% nucleotide identity with those canine RV strain, while it shared 99% identity with a human RV strain NN496-16.

From our phylogenetic analysis, almost all G3P[9] strains of animal and human origins consistently possess an A3 genotype in the NSP1 gene. This observation is not new, and it has been said that the unique combination of the VP4 P[9] and NSP1 A3 gene combination might provide these viruses with a competitive replicative capacity in various hosts [252]. Other segments of G3P[9] strain except NSP5/6 were dominated with an AU-1-like genotype constellation either from human, cat, and/or dog. An unusual DS-1-like genetic backbone from bovine were also found in a few G3P[9] strain [235]. Future studies on molecular characterization of whole genome sequencing of RV is needed in order to monitor the evolution of human and animal RV strain and to identify the potential of human-animal reassorment.

This study has several limitations. To our knowledge, this indoor house cat was not exposed to any wildlife. The household of the cat owner did not report any illness, although anthroponosis cannot be eliminated. Due to the social restrictions relating to the coronavirus pandemic and the heightened awareness of good hygiene, infection acquired from travelling was unlikely. Despite these limitations, our report of reassortant RV strain from a domestic animal may be useful in the future for epidemiological study of RV emergence and the surveillance of such viruses with zoonotic potential.

CHAPTER VII

GENERAL DISCUSSION

Summary

The aim of this study is to explore the current epidemiology and genotype diversity of RVA circulated in Thailand. To accomplish the objective, I began screening RVA using TaqMan probe one-step real-time RT-PCR assay targeted NSP3 gene. All positive RVA samples were then genotyped by conventional multiplex RT-PCR. Deep analysis by the WGS especially for the vaccine-derived and unusual strains has been done to characterize the genetic variability and potential interspecies transmission. Characterization of the antigenic differences and protein structure between circulating RVA strains after vaccine introduction in Thailand and RVA vaccines, Rotarix and RotaTeq was conducted to map the viral mutation.

During 2020 and 2021, approximately 10% of pediatric diarrhea was attributable to RV infection. The most frequent genotype detected was G3P[8]. This prevalence is lower than previous years, possibly because of social measures implemented by the Thai government to mitigate coronavirus transmission and an early impact of RV vaccine inclusion in Thailand national immunization program [37, 202]. Conventional multiplex RT-PCR was done to detect RVA mix infection and to determine the probability of genetic reassortment among RVs. The use of conventional multiplex RT-PCR in our study revealed many mixed infections with more than one

RV strain in a given sample (24%). This is a novel finding compared to our previous studies in which we mostly relied on a simple RT-PCR and had resulted in the detection bias of a single RV strain per sample [36, 37]. Using this method, I successfully detect vaccine-viral shedding in two stool samples. While we could confidently conclude that B8019 possessed genome constellation G1-P[8]-I2-R2-C2-M2-A3-N2-T6-E2-H3, multiple vaccine-like RV strains detected in B7711 combined with probable wild-type (G3P[4]) RV co-infection prevents us from assigning definitive constellation. Consistent with my study, multiple vaccine-like RV strains were also found in an Australian study in children with underlying medical condition [214]. One possibility for the derivation of G1P[8] in B8019 is the dual reassortment between two parental strains from RotaTeq. Both Rotarix and RotaTeq are live vaccines, so they can replicate and are shed in the feces after vaccination [151].

RVA of genotype G3 is one of the frequently detected strains worldwide and has broad host range [224-226]. Although G3 is often found in combination with P[8], P[4], and P[6], pairings with P[9] and P[10] are less frequently reported [115, 225, 229, 233-236]. RVA infection is traditionally associated with very young children, but occasional infection among adults does occur [36, 46]. Finding the new genome constellation G3–P[10]–I3–R3–C3–M3–A9–N3–T3–E3–H6 in diarrheic adults living in Bangkok was unexpected. Our two G3P[10] strains grouped with the bat-like MYAS33 strain. It is noteworthy that the latter has an identical genotype constellation as a bat RVA strain MYAS33 identified in 2013 in China [255], which suggests probable zoonotic origin. Interestingly, the genome constellation of our G3P[10] and several previously described global strains differed by a single gene segment, either VP4 or VP6.

Human G3P[9] strains including our B2682 share common origins with feline RVA [235, 244, 245] and are infrequently detected in humans [226, 246-250]. The B2682 possessed identical genome constellation G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H6 as another Thai strain CU365 previously reported by our group [235] and strains L621 and E2451 from China [251]. Interestingly, we also found novel genome constellation G3-P[9]-I2-R2-C2-M2-A3-N2-T3-E3-H3 of feline RVA from 2-year-old indoor female Siamese cat. The genes encoding VP4, VP7, NSP1, NSP3, NSP4, NSP5 were related to human feline AU-1-like, while the remaining genes were similar to the human bovine DS1-like and bovine RV strains. From our phylogenetic analysis, almost all G3P[9] strains of animal and human origins consistently possess an A3 genotype in the NSP1 gene. This observation is not new, and it has been said that the unique combination of the VP4 P[9] and NSP1 A3 gene combination might provide these viruses with a competitive replicative capacity in various hosts [252].

The use of rotavirus vaccines in Thailand is currently not widespread and Thai adults are extremely unlikely to have been vaccinated. When we mapped the deduced amino acid residue changes of our G3 strains onto the trimeric VP7 protein structure of the vaccineG3 strain, we identified several differences on the antigenic epitopes. Many of the differences were P type-specific, such as V129I in 7-1a for G3P[4] or D242T in 7-1b for G3P[10]. Residues of our G3P[9] differed most from the vaccine, particularly on the 7-1b antigenic epitope. Interestingly, the K238N substitution, which is also present in various G3 strains from Argentina, Belgium, Pakistan, and Lebanon [224, 225, 257-259], can potentially introduce an N-linked glycosylation in the 7-1b epitope and reduce antibody neutralization [260].

Findings in this study demonstrated that RV infection in Thailand is predominated by the G3P[8] strain. We also found possible vaccine-derived RV shedding in Thai children with diarrhea, and the observed wild-type RV infection concurrent with recent RotaTeq vaccination. Potential RV shedding among vaccine recipients will require monitoring, especially in household with many children or daycare to mitigate potential horizontal transmission of vaccine-derived RV.G3 RVA identified in this study highlights unusual RVA genotype constellations even in urban settings, which would have otherwise eluded detection without a thorough genome analysis. Some differences on the antigenic epitopes suggested mutation and potential antibody neutralization reduction.

Future Direction

Continuing surveillance and molecular characterization of rotavirus genotypes both human and animals are important to better understanding of the genetic variability, evolutionary dynamics, and possible interspecies transmission. A better understanding of virus epidemiology, genetic variability, and evolutionary dynamics plays an essential role in the success of vaccine implementation and determining future strain inclusions in the vaccine.

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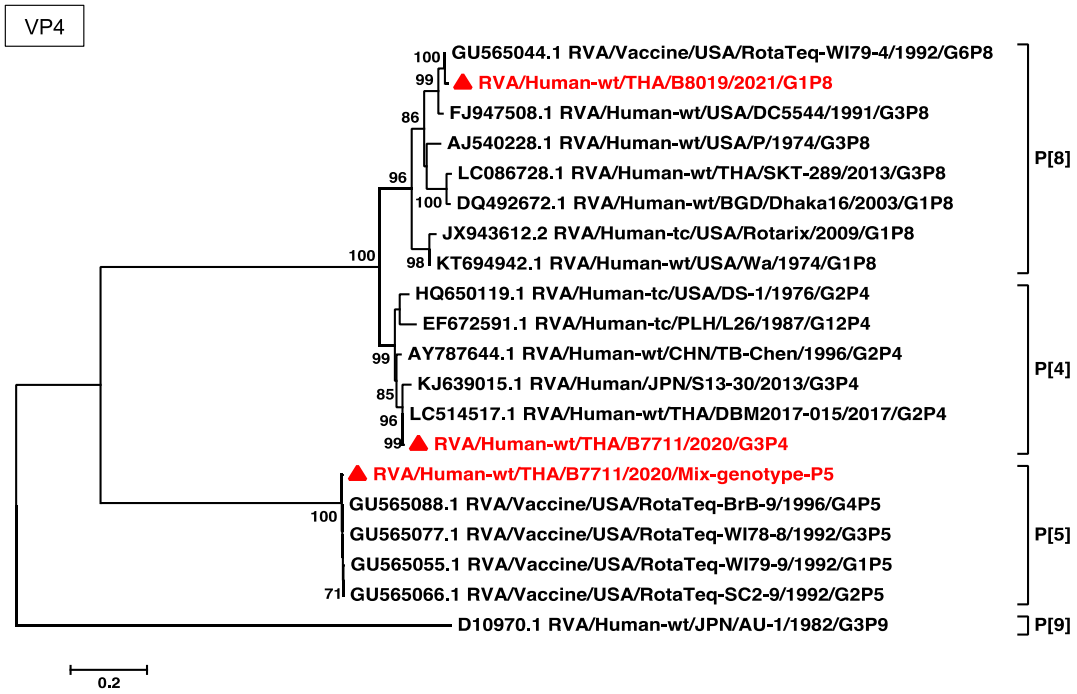
Appendix

Supporting Information Chapter IV



S1 Fig. Additional phylogenetic tree of VP4. Amplified region for different genotypes differed because of the multiplex primers used for simultaneous detection of multiple VP4 segments, which warrants this additional tree.

Supplementary Materials Chapter V

S1 Table. Oligonucleotide primers used to amplify the VP4 segment of P[10].

Primer name	Sequence (5' to 3')	Position*
VP4_P10_F2	TGGCTTCGCTCATTTACAGAC	2-22
VP4_P10_R1153	ACTGGCAATGCGAAACTGTA	1134-1153
VP4_P10_F987	TGGTGGATCATTACCAACTGAC	987-1008
VP4_P10_R2260	CTCGTAGCACTCTAGGATCAGA	2239-2260

*Relative to the MYAS33 strain (GenBank accession number KF649187).

S2 Table. Nucleotide sequence identities between 7 G3 RVA strains and their closest related strains for all gene segments.

Strain Name	Gene	Strains that exhibit close nucleotide sequence identities	Identity (%)
RVA/Human-wt/THA/B5383/2018/G3P[4]	VP1	RVA/Human-wt/THA/DBM2018-291/2018/G9P[8]	99.82%
	VP2	RVA/Human-wt/THA/DBM2017-003/2017/G2P[4]	99.96%
	VP3	RVA/Human-wt/JPN/Tokyo17-10/2017/G2P[4]	99.42%
	VP4	RVA/Human-wt/THA/DBM2018-105/2018/G2P[4]	99.86%
	VP6	RVA/Human-wt/THA/DBM2017-015/2017/G2P[4]	99.92%
	VP7	RVA/Human-wt/IDN/D009617g/2015/G3P[8]	99.90%
	NSP1	RVA/Human-wt/THA/DBM2017-003/2017/G2P[4]	99.60%
	NSP2	RVA/Human-wt/THA/DBM2018-105/2018/G2P[4]	99.90%
	NSP3	RVA/Human-wt/THA/DBM2017-003/2017/G2P[4]	99.90%
	NSP4	RVA/Human-wt/USA/SSCRTV_00011/2013/G2P[4]	99.86%
	NSP5/6	RVA/Human-wt/THA/DBM2017-003/2017/G2P[4]	99.51%
RVA/Human-wt/THA/B5356/2018/G3P[4]	VP1	RVA/Human-wt/ESP/SS96217158/2015/G3P[8]	99.51%
	VP2	RVA/Human-wt/THA/DBM2017-015/2017/G2P[4]	99.96%
	VP3	RVA/Human-wt/ESP/SS61720845/2015/G3P[8]	99.81%
	VP4	RVA/Human-wt/THA/DBM2018-105/2018/G2P[4]	100.00%
	VP6	RVA/Human-wt/THA/DBM2017-015/2017/G2P[4]	99.84%
	VP7	RVA/Human-wt/ESP/SS98242319/2015/G3P[8]	99.71%
	NSP1	RVA/Human-wt/DEU/GER33-15/2015/G3P[8]	99.66%
	NSP2	RVA/Human-wt/THA/DBM2018-105/2018/G2P[4	100.00%
	NSP3	RVA/Human-wt/THA/DBM2017-015/2017/G2P[4]	99.70%
	NSP4	RVA/Human-wt/ESP/SS61720845/2015/G3P[8]	99.33%

	NSP5/6	RVA/Human-wt/THA/DBM2018-291/2018/G9P[8]	99.75%
RVA/Human-wt/THA/B4401/2017/G3P[6]	VP1	RVA/Human-wt/IDN/STM182/2016/G3P[6]	99.85%
	VP2	RVA/Human-wt/IDN/STM169/2016/G3P[6]	99.48%
	VP3	RVA/Human-wt/THA/MS2014-0134/2014/G3P[8]	99.72%
	VP4	RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6]	99.53%
	VP6	RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6]	99.69%
	VP7	RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6]	99.90%
	NSP1	RVA/Human-wt/IDN/STM182/2016/G3P[6]	98.99%
	NSP2	RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6]	99.90%
	NSP3	RVA/Human-wt/IDN/STM169/2016/G3P[6]	99.61%
	NSP4	RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6]	100.00%
	NSP5/6	RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6]	99.86%
RVA/Human-wt/THA/B5368/2018/G3P[6]	VP1	RVA/Human-wt/IDN/STM182/2016/G3P[6]	99.85%
	VP2	RVA/Human-wt/IDN/STM050/2015/G3P[8]	99.47%
	VP3	RVA/Human-wt/THA/MS2014-0134/2014/G3P[8]	99.69%
	VP4	RVA/Human-wt/THA/CMHN49-12/2012/G12P[6]	99.05%
	VP6	RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6]	99.92%
	VP7	RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6]	99.89%
	NSP1	RVA/Human-wt/IDN/STM182/2016/G3P[6]	99.46%
	NSP2	RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6]	99.70%
	NSP3	RVA/Human-wt/IDN/STM169/2016/G3P[6]	99.22%
	NSP4	RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6]	99.16%
	NSP5/6	RVA/Human-wt/RUS/Novosibirsk/NS17-A1301/2017/G3P[6]	99.61%
RVA/Human-wt/THA/B2682/2016/G3P[9]	VP1	RVA/Human-wt/JPN/To16-11/2016/G3P[3]	98.95%
	VP2	RVA/Human-wt/JPN/To16-11/2016/G3P[3]	98.80%
	VP3	RVA/Human-wt/JPN/To16-11/2016/G3P[3]	98.45%

	VP4	RVA/Human-wt/CHN/E2451/2011/G3P[9]	98.07%
	VP6	RVA/Human-tc/THA/T152/1998/G12P[9]	98.24%
	VP7	RVA/Human-wt/JPN/To16-11/2016/G3P[3]	98.32%
	NSP1	RVA/Human-wt/JPN/To16-11/2016/G3P[3]	97.08%
	NSP2	RVA/Human-wt/JPN/To16-11/2016/G3P[3]	98.88%
	NSP3	RVA/Human-wt/JPN/To16-11/2016/G3P[3]	97.79%
	NSP4	RVA/Human-wt/RUS/NN496-16/2016/G3P[9]	97.95%
	NSP5/6	RVA/Human-wt/JPN/To16-11/2016/G3P[3]	99.14%
RVA/Human-wt/THA/B4684/2018/G3P[10]	VP1	RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	89.41%
	VP2	RVA/Rhesus-tc/USA/TUCH/2002/G3P[24]	91.32%
	VP3	RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]	88.06%
	VP4	RVA/Human-wt/THA/MS2015-1-0001/G3P[10]	96.48%
	VP6	RVA/Human-wt/CHN/M2-102/2014/G3P[3]	98.67%
	VP7	RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	96.36%
	NSP1	RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	94.09%
	NSP2	RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	97.71%
	NSP3	RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	95.53%
	NSP4	RVA/Human-wt/THA/CMH222/2005/G3P[3]	96.36%
	NSP5/6	RVA/Alpaca-wt/PER/Alp5403/2010/G3P[40]	95.74%
RVA/Human-wt/THA/B5662/2018/G3P[10]	VP1	RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	93.04%
	VP2	RVA/Rhesus-tc/USA/TUCH/2002/G3P[24]	91.45%
	VP3	RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]	88.17%
	VP4	RVA/Human-wt/THA/MS2015-1-0001/G3P[10]	96.35%
	VP6	RVA/Human-wt/CHN/M2-102/2014/G3P[3]	98.43%
	VP7	RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	96.26%
	NSP1	RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	94.43%

	NSP2	RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	96.79%
	NSP3	RVA/Bat-tc/CHN/MYAS33/2013/G3P[10]	95.43%
	NSP4	RVA/Human-wt/THA/CMH222/2005/G3P[3]	96.50%
	NSP5/6	RVA/Alpaca-wt/PER/Alp5403/2010/G3P[40]	95.33%

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AWARD RECEIVED	1. Awardee of BPPLN Scholarship from Directorate General of Higher Education, Research, and Technology; Ministry of Education, Culture, Research, and Technology of The Republic of Indonesia. 2. Awardee of High Quality Publication in Drugs and Medicine Category from the Ministry of Research, and Technology of The Republic of Indonesia.