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WHOLE GENOME CHARACTERIZATION OF G9P[4] AND G9P[8] ROTAVIRUS STRAINS IDENTIFIED IN LEBANON

BY
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AN ABSTRACT OF THE THESIS OF

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Rotavirus is considered globally the leading cause of severe diarrheal infections among children under the age of 5 years and is responsible for about 215 000 deaths annually. Rotaviruses are classified based on their outermost shell proteins, VP7 (G-genotype) and VP4 (P-genotype) with at least 36 G genotypes and 51 P genotypes identified up-to-date. Although there are common globally circulating genotypes (G1P[8],G3P[8], G9P[8],G2P[4]), the segmented genome of the virus and possible vaccine pressure might explain the emergence of unique genotypes such as G9P[4]. In Lebanon, a hospital-based study conducted between 2011-2013 revealed that 30.3% of gastroenteritis-hospitalizations were attributed to rotavirus A (RVA), with major genotypes being G1P[8] (36%) and G9P[8] (26.4%). Newly emerging, unique genotypes such as G9P[4] constituted <1%.

In this study we performed full genome characterization these unique G9P[4] strains and one G9P[8] strain in Lebanon by using virome capture technology and phylogenetic analysis. Our goal was to elucidate their origin and genetic relatedness compared to globally circulating G9P[4] and G9P[8] specimens as well as their genetic vaccine matching.

One G9P[4] specimen was of pure DS-1 like origin and the remaining two were of mixed and diverse Wa-like/DS-1-like genotypes: RVA/Human/LEB/A095/2011/G9P[4] (G9-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2), RVA/Human/LEB/H017/2011/G9P[4] (G9-P[4]-I2-R2-C2-M2-A2-N1-T2-E2-H2), RVA/Human/LEB/H199/2011/G9P[4] (G9-P[4]-I1-R1-C1-M1-A2-N1-T1-E1-H1). The RVA/Human/LEB/NG184/2011/G9P[8] was of pure Wa-like origin (G9-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1). Phylogenetic analysis revealed that the G9P[4] strains with mixed genomic constellation emerged from a reassortment events between G2P[4] and G9P[8] stains. Overall, unlike the G9P[8] strains' pure genomic backbone, multiple lineages of G9P[4] seemed to have emerged globally. The analysis indicated that Lebanese G9P[4] strains seemed to have emerged through multiple introductions or reassortment events. The G9P[8] strain shared common ancestry with Ethiopian G9P[8] providing potential clues regarding their geographic spread. Overall, the G9 strains' antigenic epitopes were highly conserved among each other but contained a large number of mutations compared to the vaccine strains. This warrants further investigations to monitor the evolution and spread of these emerging genotypes in Lebanon and globally and to determine the efficacy of current vaccines against them.

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INTRODUCTION

A. Gastroenteritis

1. Definition

Gastroenteritis is defined as the inflammation of the stomach or intestinal lining due to an infection or ingestion of drugs or toxins. Patients suffering from this disease, typically present with diarrhea, vomiting and abdominal pain (Boyce, 2017). Gastrointestinal infections can be divided into (i) non-inflammatory or infectious diarrheal diseases (ii) inflammatory gastroenteritis, which start as watery diarrhea but can result in blood or mucus filled feces and finally (iii) systemic gastroenteritis in which the pathogen has spread from the stomach to other areas in the body (Vaishanvi, 2013). Infectious gastroenteritis majorly impacts the pediatric population with about 5 billion episodes occurring worldwide annually (Cochran, 2017). Treatment of gastroenteritis is generally symptom based and therefore includes oral hydration therapy (ORT) and focus on adequate nutrition (Duggan, Santosham & Glass, 1992).

2. Etiology

Organisms causing gastroenteritis include bacteria, parasites, and viruses. The severity of the diseases caused by these pathogens varies with different age groups (de Wit et al, 2001). Bacterial gastroenteritis can be caused by *Escherichia, Salmonella* and *Campylobacter species (*O'Ryan et al, 2014*).* Parasitic agents include *Cryptosportidium, Isospora* and *Giardia (*Vaishanvi,

2013*).* Nevertheless, the leading cause of acute watery diarrhea in children under 2 years of age and the second cause in children between 24 and 60 months, is rotavirus (O'Ryan et al, 2014).

B. Rotavirus

1. Role of rotavirus in gastroenteritis

Rotavirus is considered the major cause of gastroenteritis-associated hospitalizations and deaths in children under 5 years of age (de Zoysa & Faechem, 1985; Parashar et al, 1998; Operario et al, 2017). The mortality rates due to non-rotavirus diarrhea are decreasing due to successful preventive measurements. Nevertheless, controlling rotavirus burden still seems challenging and can be explained by (i) mode of transmission of rotavirus, which is primarily person-to-person and therefore improvements in sanitation or water supplies are ineffective (ii) oral hydration therapy lacks sufficient effectiveness in rotavirus diseases due to the severe vomiting and finally (iii) no antimicrobial therapy against rotavirus infections (Parashar et al, 2006).

2. Discovery of Rotavirus

In the 1960s, Adams and Kraft observed a virus-like particle in mice intestinal tissue, in which the mice were orally administered with epizootic diarrhea of infant mice (EDIM) (Adams & Kraft, 1963). One year later, in 1973 Flewett et al, observed a 70nm thin-section electron microscopy of human rotavirus in a duodenal mucosa sample (Flewett et al, 1973). Interestingly, Flewett et al also later described virus particles in the feces of young children with acute gastroenteritis that were indistinguishable in size and shape from those in newborn calves. Since these viral particles were different from norovirus and reovirus, they were designated as rotavirus, based on their wheel like structure (Flewett et al, 1974).

3. Transmission

Rotavirus is highly contagious. The infectious dose is as low as 10 particles (Ward et al, 1986). It is mainly transmitted via the fecal-oral route; as such, the site of replication and the symptoms manifested due to a rotavirus infection are primarily gastrointestinal (Kapikain et al, 1983). The respiratory transmission mode could also play a role in rotavirus infection due to the inhalation of rotavirus infected respiratory droplets from a diseased child (Zheng et al, 1991). Zheng et al observed 25 out of 89 infants with rotavirus particles in oropharyngeal aspirates and the detection of rotavirus in the oropharyngeal aspirate was independent of the presence of rotavirus in the feces, development of diarrhea or vomiting; however, it was associated with respiratory manifestations. The incubation period of rotavirus infection is usually 2-3 days and symptoms persist for 3-8 days. Furthermore, virus shedding can start prior to the appearance of symptoms and last until after they have resolved (Richardson et al, 1998).

4. Clinical Symptoms

The major symptoms of a rotavirus infection are gastrointestinal; however, the clinical representation of the disease differs among children and adults. Although it is difficult to identify a rotavirus gastroenteritis based on the clinical symptoms alone, children typically present with high fever and more severe vomiting and diarrhea than other gastroenteritis illnesses (Coffin et al, 2006). Subsequent fluid loss could result in dehydration and electrolyte imbalance. If symptomatic treatment and supportive measures, such as fluid replacement therapies, are not available or not taken, the disease might worsen, causing seizures and eventually death (Lynch et al, 2003). Although, rotavirus RNA was detected in the spleen, lymph node, heart, lung, testes, kidney, and

bladder, there is no evidence of a direct rotavirus infection in these organs (Lynch et al, 2003). Similarly, Blutt et al discovered rotavirus antigenemia and viremia in infected mice, which could explain the presence of rotavirus in extra-intestinal cells (Blutt et al, 2006). A study by Hemming et al, reported that 61% of rotavirus infected children experienced a rotavirus antigenemia, which was associated with increased severity of fever and vomiting (Hemming et al, 2014). Nevertheless, the impact of rotavirus antigenemia on extra-intestinal symptoms, is still not well understood. In adults, the symptoms are generally less complicated and might include subclinical or no clinical manifestations mainly due to the development of immune protection against rotavirus during early childhood (Kim et al, 1977). Nevertheless, rotavirus infection in adults is underappreciated since adults with underlying health conditions might experience worsening of their underlying disease, which can be potentially fatal (Friesma et al, 2012).

5. Diagnosis

Rotavirus diagnosis is done using stool specimens (Yolken et al, 1988). Several diagnostic tools exist for rotavirus. Electron microscope (EM) represents one of the earliest methods for rotavirus identification based on the observation of virus particles with wheel-like structure (Figure 1) (Roingeard, 2012). However, this technique is time consuming and requires a high level of technical skills. This is why scientists relied on serological techniques such as immunofluorescence that proved to be more reliable, easier to perform, and faster (Yolken et al, 1977). Furthermore, methods that can detect rotavirus antigens using rotavirus group-specific monoclonal antibodies were developed, such as the sandwich enzyme-linked immunosorbent assay (ELISA) (Beards et al, 1984). More recently, rapid antigen detection kits were developed and deployed in clinical use. This technique is advantageous because it is relatively cheap, user-

friendly, equipment-free, and can be used at point-of-care (Ope et al, 2017). Nonetheless, molecular techniques such as reverse-transcriptase polymerase chain reaction (RT-PCR) are considered the gold-standard for diagnosis of rotavirus nowadays because of their timeliness and improved sensitivity and specificity (Pang et al, 2004).

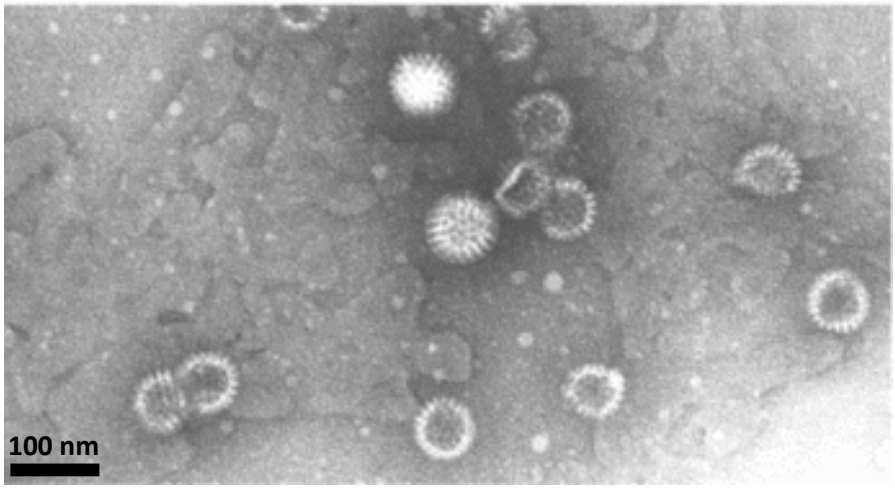


Figure 1: Electron micrograph of rotavirus (Roingeard, 2012)

C. Rotavirus structure and classification

1. Rotavirus structure

Rotavirus belongs to the family *Reoviridae.* Mature virions are non-enveloped, triple-layered in nature and harbor 11 gene segments of double-stranded RNA (Prasad & Estes, 1997). The genes code for 5 non-structural (NSP1, NSP2, NSP3, NSP4, NSP5) and 6 structural/viral-proteins (VP1, VP2, VP3, VP4, VP6, VP7) proteins (Jayaram et al, 2004). The number after each structural protein name is based on its molecular weight; VP1 being the largest. VP4 is

proteolyticaly cleaved into VP5* (stalk) and VP8* (globular head) and the latter is the smallest

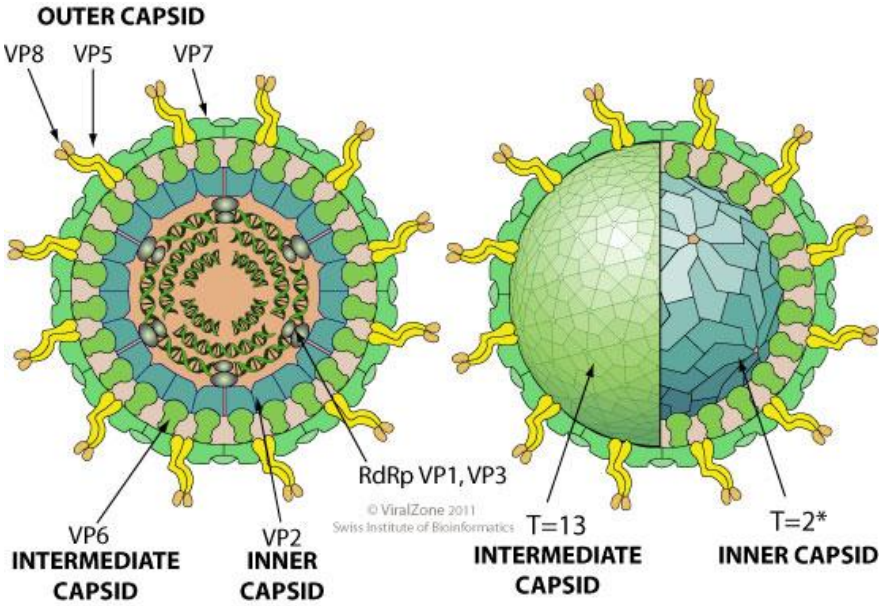


Figure 2: Rotavirus structure (Viral Zone, 2011)

among the structural proteins (Estes et al, 1981). Cryoelectron microscopy and image analysis revealed that outer and inner capsid of the virus has icosahedral lattice symmetry and the space between the capsids forms an open aqueous pathway, responsible for the passage of ions, small regulatory molecules and dsRNA genome (Yeager et al, 1990). The core consists of the dsRNA, VP1 and VP3, which make up the single-layered particle (SLP) (Figure 2). The double-layered particle (DLP) includes a thin layer of VP2 surrounded by VP6 trimers (Prasad et al, 1996; Mathieu et al, 2001). The addition of the VP4 and VP7 glycoproteins gives rise to the triple-layered particles (TLP) or the infectious virion (Shaw et al, 1993).

2. Rotavirus protein functions

The functions of the rotavirus proteins are summarized in table 1. VP2 protein binds directly to the RNA, whereas the VP1 protein is the RNA-dependent RNA polymerase. VP3 is a methyl and guanylyl transferase enzyme and is important in post-transcriptional modifications, such as 5' capping (Petrie et al, 1982: Petrie et al, 1984: Richardson et al, 1986; Bisaillon and Lemay, 1997).

VP6 protein plays an important role in virus morphogenesis by interacting with the inner layer protein VP2, and outer layer VP4 and VP7 proteins and it is also involved in RNA transcription (Pesavento et al, 2006; Blutt et al, 2007). The VP4 is an outermost dimer protein consisting of a distal globular head (VP8*) that mediates cell attachment, and a Fab binding region (VP5*) that is crucial for cell penetration. Blockage of the VP5* region by antibodies neutralizes infectivity and inhibits viral entry into the cell (Prasad et al, 1990). VP7 is a calcium binding glycoprotein that is target for neutralizing immunity (Prasad et al, 1987). In general, non-structural proteins are involved in various functions such as replication, genome packaging, and translation. For instance, NSP1 acts antagonistically on type 1 interferons, therefore promoting viral survival by inhibiting antiviral immune responses (Barro and Patton, 2005). NSP2 is an NTPase and helps in genome replication and packaging (Afrikanova et al, 1998), whereas NSP3 is detrimental in viral mRNA translation (Piron et al, 1998). NSP5 protein is a protein kinase (Mattion et al, 1991). The NSP4 protein has a dual function by contributing to viral morphogenesis (Tian et al, 1996) and functioning as an enterotoxin that triggers the typical gastrointestinal symptoms (Ball et al, 1996).

Table 1: Rotavirus protein functions

PROTEIN(S)	FUNCTION(S)
VP1	RNA - dependent RNA polymerase
VP2	RNA binding
VP3	Guanylyl and methyl transferase
VP6	Extra - intestinal spread
VP4	Virulence and viral entry
VP7	Rough endopasmic reticulumintegral membrane glycoprotein & viral entry
NSP1	Antagonist of interferon response
NSP2	Genome replication/packaging & NTPase
NSP3	Viral mRNA translation & shuts off host protein synthesis
NSP4	Viral enterotoxin & viral morphogenesis
NSP5/NSP6	Protein kinase & constituent of viroplasm

3. Rotavirus replication cycle

Rotavirus surface glycoprotein VP4, mediates binding and attachment to cell surface receptors via its VP8* subunit (Ruiz et al, 2009). Studies have demonstrated that the viral hemagglutinin, VP8*, binds to sialic acid receptors via the induced-fit model and thus mediates initial cell attachment (Dormitzer et al, 2002). In support of these observations, researchers showed that treatment of sialidase-sensitive strains with sialidase, reduced binding (36%) and infection (77%) (Haeselhort et al, 2009). Nevertheless, a new region in the VP8* structure that is similar to the carbohydrate-binding region of galectin might be responsible for carbohydrate binding in sialidase insensitive rotavirus strains (Blanchard et al, 2007). Interestingly, some human rotaviruses have the ability to bind to human histo-blood group antigens. As such, a study has shown that out of 85 children tested positively for rotavirus, all were either human histo-blood group antigens secretors or partial secretors (Trang et al, 2014). Following binding, the virus is then internalized via calcium-dependent endocytosis (Ruiz et al, 2000). Through simple diffusion or active transport mechanisms, calcium (Ca^{2+}) ions start to exit the endocytic vesicle into the cellular compartment (Gerasimenko et al, 1998). Once endosomal and cytoplasmic calcium levels equilibrate, disruption and release of VP7 occurs followed by destabilization of VP4 protein. The VP5* will interact with the endosomal membrane and mediate fusion and pore formation, allowing the DLP to exit to the cytoplasm as illustrated in Figure 3 (Ruiz et al, 1997; Crawford et al, 2017). Once in the cytoplasm, the DLP is transcriptionally activated to transcribe mRNA, which will eventually be translated into viral proteins in the cytoplasm (Cohen et al, 1979; Charpilienne et al, 2002). When the number of translated proteins exceeds threshold, they will accumulate in the viroplasm where synthesis of viral RNA genome occurs and finally the DLP assembly (Silvestri et al, 2004). The newly synthesized DLPs bud into the endoplasmic reticulum (ER), a process that is facilitated by binding

of NSP4 to the ER. As a result, the DLPs acquire a "pseudoenvelope" derived from the ER membrane (Altenburg, Graham and Estes, 1980). In the ER, the high Ca^{2+} concentrations mediate virus maturation, which is completed with the acquisition of the VP7 and VP4 proteins and the loss of NSP4 and the "pseudoenvelope" (Ruiz, Cohen and Michelangeli, 2009). Release of the progeny virions occurs via cell lysis or Golgi-independent non-classical vesicular transport (Crawford et al, 2017).

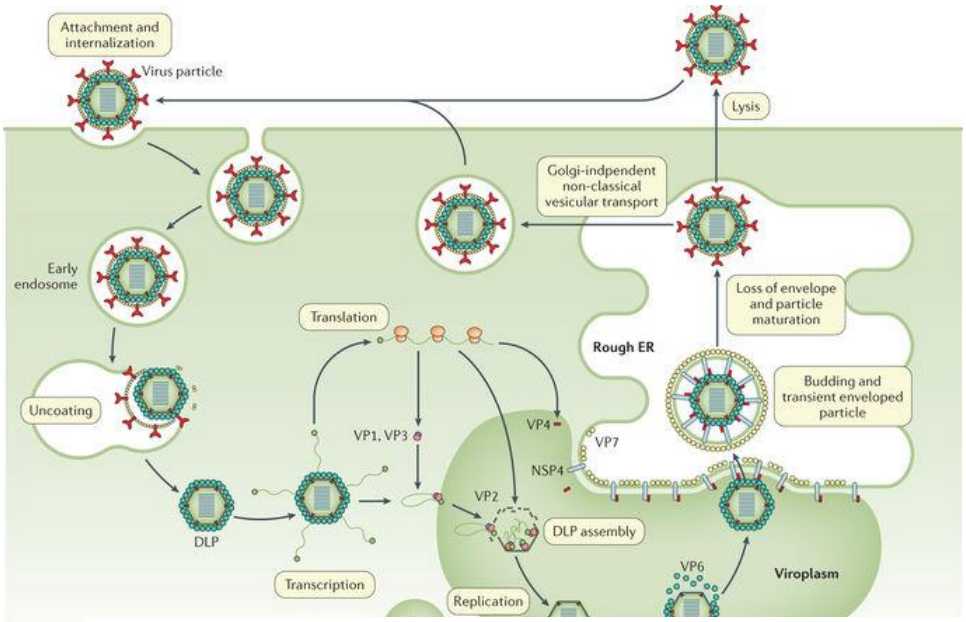


Figure 3: Rotavirus replication cycle (Crawford et al, 2017)

4. Pathogenesis

The pathogenesis of rotavirus-induced diarrhea is complex and involves a variety of factors. Viral entry into the intestinal enterocytes and the subsequent viral replication leads to an increase in Ca^{2+} concentrations. This increase in Ca^{2+} ions disrupts the cytoskeleton and tight junctions and thus harms the integrity of the enterocytes (Ruiz et al, 2000). Destruction of the enterocytes leads to malabsorption and subsequent diarrhea (Kapikian, Hoshino and Chanock, 2001). The viral enterotoxin (NSP4) can trigger intracellular remodeling of actin and thus possibly cause disassembly of the microvilar F-actin, leading to destabilization of the enterocytes (Brunet et al, 2000). Furthermore, NSP4 triggers increase in the calcium efflux from the ER by activating the phospholipase-C (PLC) pathway and activates the enteric nervous system (Dong et al, 1997). The latter results in the release of neurotransmitters that will increase gastrointestinal motility and activate the vomiting centers in the medulla oblongata of the brainstem (Lundgren et al, 2003).

5. Rotavirus classification

The International Committee on Taxonomy of Viruses (ICTV) have classified rotavirus into 10 groups (A-J) based on the amino acid sequences of the VP6 protein (Crawford et al, 2017; Banyai et al; 2017; Mihalov-Kovacs et al, 2015; Matthijnssens et al, 2012). Groups A, B and C usually cause infections in humans and animals, whereas groups D, E ,F and G infect animals (Estes and Kapikian, 2007). Rotavirus group A is the most common cause of human infections (Li et al, 2016). Reassortment events are more likely to occur within the same group than across different groups, thus increasing the diversity of a certain rotavirus group (Yolken et al, 1988). Further, rotavirus is designated into two subgroups (1 and 2) based on the antigenic domains of the VP6 protein (Estes and Kohen, 1989; Lopez et al, 1994). Each subgroup can be further

classified into serotypes on the basis of the outermost shell proteins, VP7 and VP4 (Kalicia et al, 1983; Kalicia et al, 1981). Neutralization tests using cell culture as well as indirect immunofluorescence, allowed the differentiation of rotavirus strains into various serotypes (Bears et al, 1980). The advent of molecular techniques including sequencing motivated scientists to shift from serotype to genotype; the latter is based on the genetic changes within the VP7 and VP4 segments. (Gentsch et al, 1992; Lopez-Tort et al, 2015).

6. Rotavirus genotyping

A binary classification is used to designate rotaviruses into G and P genotypes based on the genetic diversity of VP7 **g**lycoprotein and the VP4 **p**rotease-sensitive protein, respectively (Hoshino and Kapikian, 1996). In addition, a more recent, full genome-based classification system was developed to distinguish the genotype of every genome segment with the following nomenclature: Gx–P[x]–Ix–Rx–Cx–Mx–Ax–Nx–Tx–Ex–Hx, representing the genotypes of VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5, respectively, with x being an integer (Matthijenssens et al, 2011). These can be further classified into three major groups. The Wa-like genotype of porcine-origin is represented by I1-R1-C1-(…) and includes G1P[8], G3P[8],G4P[8],G9P[8] (…). The DS-1-like strains having bovine-origin, possess I2-R2-C2-(…) including among others G2P[4]. Finally, representing the minority of strains is the AU-1-like with feline origin, I3-R3-C3-(…) and includes G3P[9] genotype (Matthijnssens and Ranst, 2012). There are so far 36 G genotypes and 51 P genotypes (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg) with G1, G2, G3, G4 and G9 in combination with either P[4] or P[8] being the most common genotypes associated with human infections (Banyai et al, 2012).

D. Rotavirus burden

Parashar et al estimated that between 1986 through 2000 there were approximately 111 million episodes of diarrhea, 25 million clinic visits, 2 million hospitalizations and 440.000 deaths each year in children under the age of 5 years (Figure 4) (Parashar et al, 2003). The World Health Organization (WHO) estimated that rotavirus resulted in 215,000 deaths in 2013 (Tate et al, 2016), indicating a decrease of almost 50% since 1986-2000. Furthermore, data by the WHO indicated that in 2013, the majority of global rotavirus mortality, occurred in India with 47,100 cases accounting for 22% of all rotavirus deaths, followed by Nigeria with 14%, Pakistan with 7% and Democratic Republic of the Congo with 6% (Tate et al, 2016). In Latin American and Caribbean countries, an estimate of 10 million rotavirus-associated diarrhea episodes, 1 million medical visits, and 75,000 hospitalizations were recorded in 2004 (de Oliveira, 2015). Nevertheless, the introduction of the vaccine into these countries significantly decreased rotavirus burden. As such, after the introduction of the vaccine in Latin America in 2015, the mortality rate of rotavirus decreased by 22-41% and rotavirus hospitalizations decreased by 59-81% (Desai et al, 2011). Such data emphasize the efficacy of rotavirus vaccines on decreasing rotavirus burden.

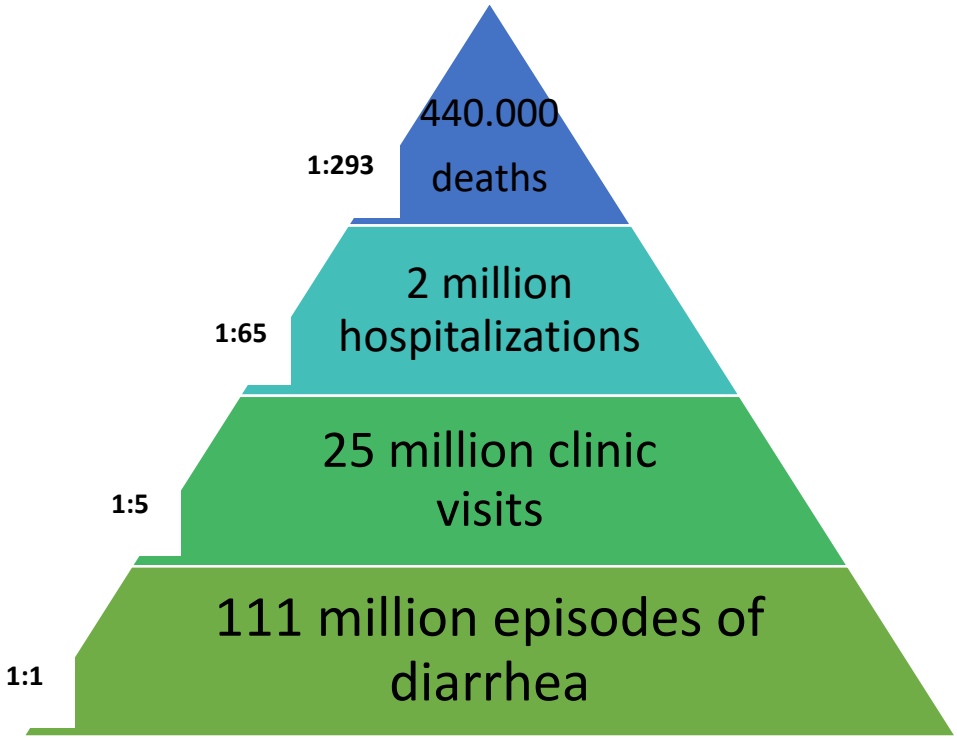


Figure 4: Rotavirus burden of disease (adapted from Parashar et al, 2003)

E. Regional and Local Burden of Rotavirus

1. Epidemiology in the Middle Eastern and North African (MENA) region

Rotavirus constitutes a major burden in the Middle East and North Africa region (MENA) accounting for 14-33 % of in- and outpatient gastroenteritis-associated visits of children-aged 2-6 years. Overall, G2P[4] was the most common genotype in 19 out of 24 MENA countries followed by G1P[8] and G9P[8] (Zaraket et al, 2017). Furthermore, G1P[8] represented 22.2% of all rotavirus positive specimens in Turkey during 2009-2010. This was followed by G1P[4] with 17.33%, G2P[4] accounting for 13.8% and finally G9P[4] and G9P[8] strains constituting 6.3% and 4.8%, respectively (Yarkin et al, 2016). In the same time frame in the UAE, acute gastroenteritis and rotavirus-associated gastroenteritis hospitalizations accounted for 12% and 6%,

respectively. Furthermore, G1P[8] was the major genotype with 56.3% out of all rotavirus strains (Howidi et al, 2014). In Tunisia, the frequency of rotavirus group A between 2009-2014 was approximately 24% and out of those tested positive for rotavirus A, G1P[8] accounted for 34.4%, G3P[8] 16.3% and finally G9P[8] 10.3% of cases (Moussa et al, 2016). Recently, a newly emerging rotavirus G6P[6] was associated with 30% of gastroenteritis cases in Africa, shedding, the light on the risk for emergence of new rotavirus genotypes (Lekana-Douki et al, 2015). Furthermore, Azaran et al published data from 2011-2012 in Iran showing that G1P[8] (80%) and G2P[4] (20%) were the major strains among RVGE cases, which constituted 36.5% of all GE cases (Azaran et al, 2016). Finally, a recent paper in Turkey investigated rotavirus-positive cases between 2014-2016 and found that the most common genotypes were G1P[8] (24.6%), G3P[8] (19.6%), and G9P[8] (12.2%). (Durmaz et al, 2017).

2. Epidemiology in Lebanon

In Lebanon, limited research exists on rotavirus. The first set of data came from Al- Ali et al in 2011, whereby rotavirus was found to account for 48% of diarrhea cases between April to May 2010. The median age was 8.5 months and the most prevalent symptoms were diarrhea and vomiting. The majority of these cases happened in Tripoli, northern Lebanon and Minyeh-Danniyeh regions. However, this study did not perform genotyping of the circulating strains and was performed over the span of two months only which might explain the relatively high prevalence of rotavirus (Al-Ali et al, 2011). Another study by Dbaibo et al from 2011-2012, reported that 27.7% of acute gastroenteritis cases in children under 5 years of age were caused by rotavirus (Dbaibo et al, 2013). Additionally, a hospital-based study conducted between 2011 and 2013 reported that rotavirus was responsible for 30.3% of gastroenteritis-associated

hospitalizations among Lebanese children under 5 years old and the peak activity was seen during the winter season (Ali et al, 2016). The most common genotypes were G1P[8], G9P[8], and G2P[4] constituting 36%, 26.4%, and 17.8% of all positive rotavirus strains, respectively. Several unique or rare genotypes represented less than 1 % of the cases, and included G9P[4] strains. The emergence of this genotype in Lebanon and globally warrants in-depth genome analysis and a better understanding of its geographic spread.

F. Rotavirus geographic distribution of G9P[4] and G9P[8]

The G9P[4] genotype has been reported in various countries in 4 continents at a frequency ranging between 0.11-80% (Table 2) (Shareen et al, 2015; Chitambara et al, 2014; Doan et al, 2017; Satish et al, 2017). The highest prevalence (36% - 80%) of rotavirus G9P[4] was reported in Latin America (Mexico, Guatemala, Honduras, and Brazil) and Japan. Although, it must be noted that these studies analyzed a small number of samples (<50 per country) and thus might not reflect the true prevalence of this genotype. While in Asia, Europe, and South America, the frequency of the G9P[4] genotype ranged between 0.11%-14.9%. A trend towards increasing prevalence of G9P[4] was noted globally.

G9P[8] genotype was found at a frequency between 0.5%-87.5% in 5 continents (Table 3) (Chieochansin et al, 2016; Tiku et al, 2014; Kim et al, 2013; Meyer et al, 2008). In a global study including 56 countries in 5 continents G9P[8] genotype presented in 4.1% of all rotavirus positive strains (Santos et al, 2005). The region that seems to constitute the majority of G9P[8] strains is India, ranging between 4.9%-15.3% from 2000 to 2012 (Tiku et al, 2014; Samajdar et al, 2008; Chitambar et al, 2014). G9P[8] strains dominated in Poland between 2005-2007 (Meyer et al,

2008). G9P[8] strains represent a similar geographic spread as the G9P[4] genotype, although the latter is less frequent.

Table 2: Geographic distribution of globally circulating G9P[4] strains

Country	Frequency	Year	Reference
India	2.60%	2009-2011	George et al. Intern J of Scientific Study. 2015
	10.10%	2011-2012	Chitambara S et al. Vaccine.2014
	8%, 11.5%, 24%	2011, 2012, 2013	Doan Y et al. Infection, Genetics, Evolution. 2017
	28.60%	2012-2013	Satish S et al. Indian J of Med Microbiol. 2017
	5.3%	2014-2015	Selvarajan S et al. Indian J Med Microbiol. 2017
	11 Kol	2011-2013	Doan Y et al. Infection, Genetics, Evolution. 2017
	42%	Jan – June 2016	Babji S et al. Vaccine. 2017
Mexico	61.50%	2010	Yen C et al. J of ID. 2011
Mexico	80%	2009 – 2010	Quaye O et al. Emerging Infectious Diseases. 2013
Guatemala	66%		
Honduras	36%		
Japan	35.30%	2011-2012	Yamamoto S et al. J of Medical Virology. 2015
Ghana	1.4%; 1.3%; 15.7%	2013; 2015; 2016	Lartey B et al. Vaccine. 2018
Brazil	14.90%	2005-2006	Munford V et al. J of ID. 2009
Italy	13.60%	2016-2017	Ianiro G et al. Infection, Genetics, Evolution. 2018
Turkey	6.30%	2009-2010	Yarkın F et al. J Immunol Clin Microbiol. 2016
Kenya	4.0%	2009-2014	Wandera E et al. J Med Virol. 2016
Korea	3.0%	Jan – Jun 2013	Kim M et al. Korea Centers for Disease Control and Prevention. 2014
Nepal	2.6%	2010	Giri S et al. Vaccine. 2018
Pakistan, Lahore	2.27%	2008-2009	Alam M et al. PLOS ONE. 2013
Denmark	1.20%	1998-2002	Fischer T et al. J Clin Microbiol. 2004
Canada	0.77%	2007-2010	McDermid A et al. BMC ID. 2012
UK, London	0.11%	2006-2007	Gomara M et al. Epidemiological Infection. 2010
	0.19%	2007-2008	
	0.24%	2008-2009	
USA	1 LB1562	2010	Lewis J et al. American Society for Microbiology. 2014

Table 3: Geographic distribution of G9P[8]

Country	Frequency	Year	Reference
India	4.9%	2000-2007	Tiku V et al. Vaccine. 2014
	5.4%	2007-2012	
	G9 (10.1 %), P[8] (54.4%), P[4] (31.4%), G9P[8] (8.6%)	2005-2006	Samajdar S et al. J of Clin Virol. 2008
	15.3%	2009	Chitambar S et al. Vaccine. 2014
	2.3%	2012-2013	Nayak et al. Archieves of Virol. 2018
6 European countries (Czech Republic, Germany, Italy, Poland, Spain, UK)	87.5 % Poland (majority)	2005-2007	Meyer N et al. ESPID. 2008
Thailand	79.1%	2015-2016	Chan-It & Chanta. J Med Virol. 2017
China	58.2%	2015	Kang & Cai. Intervirology. 2015
Argentina	54.1%	2006-2007	Stupka J et al. Infect, Gen, Evolution. 2009
Sweden	42.9%	2001-2002	Rubilar-Abreu E et al. J of Clin Microbiol. 2005
Hungary	42.8%	2012	Doro R et al. Infection, Genetics, Evolution. 2011
Latin America	29%	2001-2003	Linhares A et al. Oxford Journals. 2006
France	32.1%	2014-2015	Kaplon J et al. Clin Microbiol Inf. 2018
USA, Detroit	G9 (39.5%) , P[8] (66.5%), G9P[8] (20.6%)	2007-2009	Abdel-Haq N et al. J of Med Microbiol. 2011
Sri Lanka	17.9%	2009-2015	Palihawadana P et al. Vaccine. 2018
France	G9 (27.4%), P[8] (92.9%)	2006-2009	De Rougemont A et al. Pediatr Infect Dis.2011
Ethiopia	11.5%	2015-2016	Gelaw A et al. Infect, Gen, Evolution. 2018
Egypt	10.8%	2015-2016	Allayeh K et al. Arch Pediatr Infect Dis. 2018
Korea	9.7%	2002-2003	Jeong H et al. Clin Microbiol and Infect. 2011

Russia	6.6%	2009-2014	Kiseleva V et al. Virologica Sinica. 2018
Global	4.1%	1989-2004	Santos N et al. Rev Med Virol.2005
Thailand, Bangkok	0.5%	2012	ChieochansinT et al. Infection, Genetics, Evolution. 2016

G. Immunological mechanisms underlying a rotavirus infection

Studies have suggested that transplacental immunity plays an important role in the protection from infectious pathogens (Kohler and Farr, 1966). However, this immunity is only short lived and after approximately 3 months the child is susceptible to new infections until the age of 2 years (Bishop et al, 1983). As shown in figure 5, the probability of the first rotavirus infection increases with age during the first 2 years of life. However, the probability and severity of a subsequent infection decreases (Velázquez et al, 1996). In fact, research has shown that children who naturally acquired two infections were almost 100% protected against a moderate-to-severe rotavirus disease (Velázquez et al, 1996). Almost 50% of the children who acquired a third infection were either asymptomatic or experienced a mild illness. Therefore, these observations formed the basis for the design of the vaccination strategy for rotavirus. It was thought that if two vaccine doses were administered at a 4-8 week interval during early childhood, a protective immune response should be developed against a third infection. As a result, children

should be 100% protected from moderate-to-severe diseases and 50% protected from a rotavirus infection.

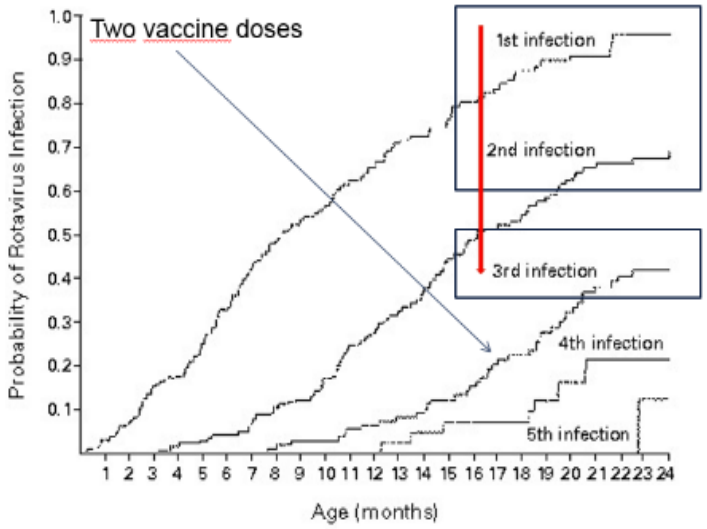


Figure 5: Probability of rotavirus infection upon repeated exposure in children (adapted from Velázquez et al, 1996| Reduction in the probability of rotavirus infection was used as a principle for rotavirus

H. Rotavirus vaccines

There are currently two live attenuated rotavirus vaccines that are administered orally. These vaccines stimulate local immunity and duplicate the natural route of rotavirus infection (Glass et al, 2005). The development of rotavirus vaccines started with the usage of animal strains that were naturally attenuated in humans, such as bovine G6 and modified WC3 bovine G6 strains. This Jennerian approach however, did not evoke an innate immune response (Vesikari et al, 1984; Clark et al, 1988; de Mol et al, 1986; Christy et al, 1988). Consequently, Rotashield vaccine, which consisted of a rhesus-human reassortant rotavirus, was developed and introduced for use in children. However, it was soon removed from the market due to increased risk of intussusception in association with vaccine administration (Bernstein et al, 1995; Hochwald and Kivela, 1999; Dennehy et al, 2008).

These failures lead to the development of the current vaccines, Rotarix and RotaTeq. Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium), a monovalent (G1P[8]) vaccine, is given at the ages of 2 and 4 months (Ward and Bernstein, 2009). While, RotaTeq (Merck & Co. Inc., West Point, PA, USA) vaccine is a pentavalent vaccine consisting of G1, G2, G3 and G4 of human origin and P[8] of bovine origin and is administered in three doses at the ages of 2, 4 and 6 months (Heaton and Ciarlet, 2007). The minimum age for administering both vaccines is 6 weeks (WHO, 2013; Ward and Bernstein, 2009; Heaton and Cialet, 2007). Both vaccines have been shown to provide heterologous protection as well, whereby Rotarix can protect against G3P[8], G4P[8] and G9P[8] viruses (Steele et al, 2012) and Rotateq can also protect against G9 strains (El Khoury et al, 2011). The efficacy and safety of these vaccines has been well demonstrated. As such Rotarix protected 85% against rotavirus gastroenteritis and rotavirus associated hospitalizations and 100% against more severe rotavirus gastroenteritis. Similarly, RotaTeq protected up to 98% against severe rotavirus gastroenteritis and reduced clinic visits for G1-G4 rotavirus genotypes by 86% (Ruiz-Palacios et al, 2006; Vesikari et al, 2006). Therefore, these vaccines were licensed and globally implemented since 2006 (WHO, 2013). Recent data suggest there are 82 countries who have introduced rotavirus vaccines into their national immunization programs (Kirkwood et al, 2017). The introduction of vaccines resulted in a significant decrease in rotavirus overall burden; nearly 40.000 to 50.000 hospitalizations were prevented in the United Stated (CDC, 2016). In Africa, 31 out of 47 Member States had introduced rotavirus vaccination by December 2016. The overall rotavirus vaccine coverage in these states was 77%, which was associated with a significant decline in pediatric diarrheal hospitalizations (Jorba et al, 2017).

In addition to the currently available vaccines, other vaccine candidates have been developed or are under development (Kirkwood et al, 2017). As such, a naturally occurring

G9P[11] reassortant between bovine P[11] gene and human rotavirus genes was isolated from an Indian child in Delhi, India. The strain was named 116E and the Indian rotavirus vaccine ROTAVAC™ (Bharat Biotech International Ltd., Hyderabad, India) was introduced by the government into the Universal Immunization Program (UIP) in 2015 after successful safety and efficacy trials (Bhandari et al, 2014). In addition to that, a bovine-human reassortant pentavalent (G1, G2, G3, G4 and G9 serotypes) rotavirus vaccine (BRV-PV) was recently developed in in India and has passed phase 3 clinical trials. This vaccine demonstrated 90% efficacy against severe RVGE caused by G1, G2, G3, G9 and G12 rotavirus strains and was found to be safe and cost-effective (Prasad et al, 2017). Furthermore, in Melbourne, Australia a human rotavirus strain G3P[6], RV3 that was isolated from the stool of asymptomatic newborns provided protection against subsequent rotavirus disease (Bishop et al, 1993). This vaccine passed phase 1 as well as early phase 2 trials and is the only strain covering the unique VP4 P[6] genotype which is becoming prevalent in Africa and Asia.

CHAPTER 2

AIMS OF THE STUDY

Major aim

To characterize the full genomes of G9P[4] rotavirus A specimens identified in Lebanon between 2011-2013.

Sub-aims

- To evaluate the geographic distribution and relatedness of the Lebanese G9P[4] specimens to globally representative G9P[4] strains.
- To assess the relatedness of the unique Lebanese G9P[4] specimens with the more common G9P[8] viruses from Lebanon and worldwide.
- To assess the genetic vaccine matching of the Lebanese G9P[4] and G9P[8] strains.

CHAPTER 3

MATERIALS AND METHODS

A. Specimens

The specimens, RVA/Human/LEB/H017/2011/G9P[4] (H017), RVA/Human/LEB/H119/2011/G9P[4] (H119), RVA/Human/LEB/A095/2011/G9P[4] (A095), and RVA/Human/LEB/NG184/2011/G9P[8] (NG184) were identified during a prospective hospital-based epidemiologic surveillance in children under 5-year-old hospitalized for gastroenteritis. The study was conducted in seven medical centers in Lebanon during January 2011 through June 2013 (Ali et al, 2016).

B. Ethical statement

Sample collection was conducted according to Good Clinical Practice and the 1996 version of the Declaration of Helsinki and was approved by the institutional review board (IRB). The specimens were collected upon obtaining a written informed consent from the parents or guardians. Participants' numbers were assigned sequentially to all children and data collection and analysis were performed respecting participants' autonomy and anonymity.

1. RNA extraction

Total RNA extraction was performed on 10% stool suspensions containing 0.5g of the fecal sample to 5mLof NaCl solution (0.89%) using the QIAmp® Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's spin column protocol. RNA was eluted in 45 µl RNase-free distilled water and stored at -20̊C until further analysis.

2. Virome Capture sequencing

Virome capture was performed in collaboration with Dr. Ian Lipkin at Columbia University Mailman School of Public Health as previously described.The strengths of VirCapSeq – VERT rely in its sensitivity and specificity, which allow the detection of the complete genome sequence of novel viruses and viral variants with a 100- to 10,000-fold increase on target reads compared to other high-throughput sequencing techniques such as conventional virome enrichment Illumina sequencing (Briese et al, 2015). Briefly, the RNA extract was reverse transcribed using SuperScript III (Thermo, Fischer) with random hexamers and the cDNA was treated with RNase H using Klenow fragment (New England Biolabs) a double-stranded cDNA/DNA was created, which was sheared to a fragment size of 200bp using Covaris E210 focused ultrasonicator. The product was purified using AxyPrep. The libraries were synthesized using KAPA library preparation kits (KAPA) with Roche/NimbleGen adapter kits, which include a library of oligonucleotides designed to target the genomes of all reported rotaviruses known to infect animals and humans. The quality and quantity of the libraries were checked using a Bioanalyzer (Agilent). Next, the libraries were mixed with SeqCap HE universal oligonucleotide, SeqCap HE index oligonucleotides, and COT DNA and vacuum evaporated at 60˚C for approximately 40 minutes. A hybridization mix was prepared using the dried samples and 2X hybridization buffer and hybridization component A (Roche/NimbleGen) prior to denaturation at 95˚C for 10 minutes. After the VirCap probe library was added and hybridized in a standard PCR thermocycler, the SeqCap Pure capture beads were mixed with the hybridization mix. The streptavidin capture beads complexed with biotinylated VirCapSeq-VERT probes were trapped (DynaMag-2 magnet; Thermo, Fischer) and washed with buffers of increasing stringency. Finally, the suspended beads were subjected to post-hybridization PCR and the PCR products were purified (Agencourt Ampure

DNA purification beads; Beckman Coulter, Brea,CA, USA) and quantitated by Bioanalyzer (Agilent). The purified product was sequenced by using the Illumina HiSeq 2500 platform (Illumina) (Briese et al, 2015).

3. Sequence Analysis

The quality of the sequencing reads was assessed by using the quality control application for high throughput sequence data implemented in FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/download.html). A total of 74,627,595, 2,997,752, 85,237,647 and 21,685,310 sequences were obtained for the specimens H199, A095, H017 and NG184, respectively. All samples were of good quality according to the FastQC analysis report (Figure 6). The reads were then imported into Geneious 11.0.2 for further processing and sequence assembly. Trimming was performed to remove low quality sequences and the reads were assembled using the map-to-reference tool and a reference rotavirus genome, which was obtained from GenBank. The consensus sequence for each genome segment was then manually edited. The BLAST tool was used to search the database for the closest reported rotavirus genome based on the sequence identity. The Virus Pathogen Database and Analysis Resource (VIPR) (https://www.viprbrc.org/brc/rvaGenotyper.spg?method=ShowCleanInputPage&decorator=reo) was used to determine the genotype of each genome segment. Nucleotide and amino acid sequence similarity matrices were calculated using the BIOEDIT software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

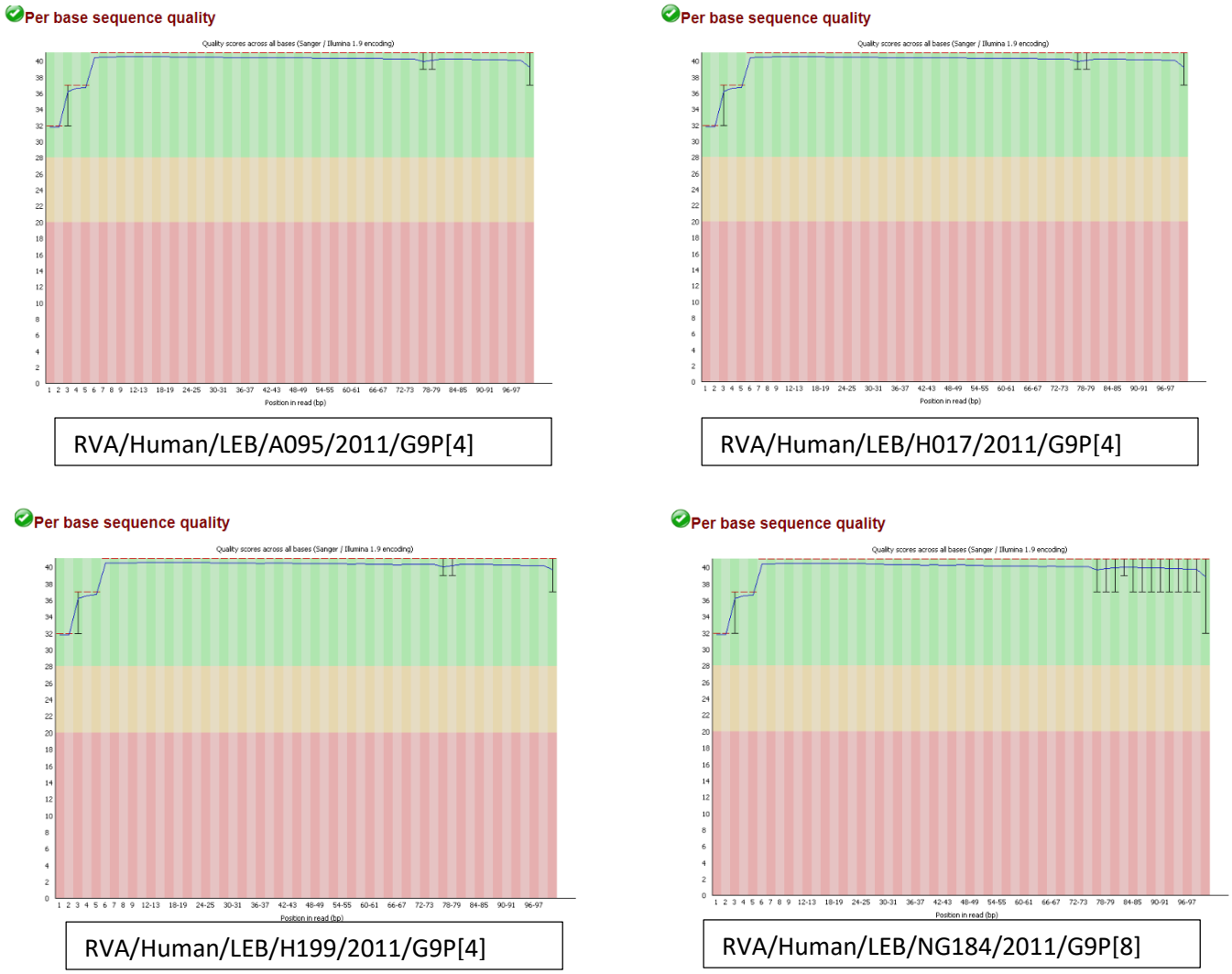


Figure 6: FastQC analysis report based on "per base sequence quality" of the Lebanese samples

4. Phylogenetic Analysis

Individual gene segments of our specimens were aligned using CLUSTAL W alignment tool in BIOEDIT software with 1) genotypically similar strains obtained from the NCBI virus variation online tool (https://www.ncbi.nlm.nih.gov/genomes/VirusVariation/Database/nph-select.cgi?taxid=28875), 2) closest BLAST hits and 3) reference strains obtained from the literature, in addition to Wa-like and DS-1 like rotavirus A prototype strains. Maximum likelihood

(ML) phylogenetic trees were constructed on the basis of the best fit nucleotide substitution model for each gene as implemented in MEGA 7.0 (Kumar et al, 2016).

The Tamura 3-parameter model with gamma distribution (T92+G) was used for VP7, NSP2, NSP3, NSP4, NSP5, VP1 and VP6, Hasegawa-Kishino-Yano model with gamma distribution (HKY+G) for VP4, General Time Reversible with gamma distribution (GTR+G+1) for NSP1, VP3, and Tamura-Nei's model with gamma distribution (TN93+G) for VP2.

Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A non-parametric bootstrap sampling analysis with 1000 replicates of the ML tree was applied using the best nucleotide substitution model.

CHAPTER 4

RESULTS

A. Patient History

The four specimens included in this study were obtained from patients aged 0.5 – 4 years and all had severe gastroenteritis as indicated by their vesikari scores, which ranged between 11-18. Vesikari clinical severity scoring system identifies the severity of a rotavirus gastroenteritis based on the clinical presentation profile. A score above or equal to 11 out of 20 belongs to the category "severe" (Lewis, 2011; Ruuska & Vesikari, 1990). The average duration of hospitalization was 4 days and three out of the four patient experienced high grade fever before being administered to the hospital. Furthermore, one of the patients harboring the H017 G9P[4] rotavirus strain, was has previously received two doses of RotaTeq vaccine.

B. Genotypic constellation of strains

The full genome constellation of human rotavirus A strains of NG184, H199, H017 and A095 were assigned as G9-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 (NG184), G9-P[4]- I1-R1-C1-M1-A2-N1-T1-E1-H1 (H199), G9-P[4]- I2-R2-C2-M2-A2-N1-T2-E2-H2 (H017) and G9-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2 (A095). The complete genotype constitution of the study strains were compared with different globally circulating G9P[8], G9P[4] and non-G9 strains (Figure 7). NG184 G9P[8] possessed a pure Wa-like genome constellation which was common for G9P[8] strains reported globally. G9P[8] strains with DS-1 like or mixed constellation were also reported, albeit rare. A095 G9P[4] exhibited DS-1 like genome constellation which was previously reported in the USA and Bangladesh. H199 and H017 G9P[4] strains showed mixed genomic constellations

that were not observed in any of the previously reported G9P[4] strains. As such, H199 constituted of 10 genes with Wa-like origin and 1 gene with a DS-1 like origin, whereas H017 contained 10 genes of DS-1 like origin and 1 gene of Wa-like origin. Based on our analysis and previously reported data the G9P[4] genome seemed to be more flexible for reassortment accommodating genes from diverse backgrounds. G9P[4] with mixed backgrounds including 1-2 genes from diverse origins on the backbone of a DS-1 like constellation were reported in India, Japan, Paraguay, and Hungary.

Strain	Genotype										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Hu-tc/USA/Wa/1974/G1P1A[8]	G1	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Hu-tc/USA/DS-1/1976/G2P1B[4]	G1	P4	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human/LEB/ H199/2011/G9P[4][a]	G9	P4	I1	R1	C1	M1	A2	N1	T1	E1	H1
RVA/Human/LEB/ H017/2011/G9P[4]	G9	P4	I2	R2	C2	M2	A2	N1	T2	E2	H2
RVA/Human/LEB/ A095/2011/G9P[4]	G9	P4	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human/LEB/NG184/2011/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BEL/B3458/2003/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BGD/Bang-062/2009/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/CHN/km15007/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/ETH/MRC-DPRU842/2012/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/HUN/ERN5009/2012/G9P8	G9	P8	I1	R1	C1	M2	A1	N1	T1	E1	H1
RVA/Human-wt/ITA/ASTI23/2007/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/ITA/AV21/2010/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Hu-wt/ITA/AV28/2010/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/ITA/JES11/2010/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/JPN/To14-30/2014/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/JPN/UR14-16/2014/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/MOZ/21155/2011/G9P[8]	G9	P8	X								
RVA/Human-wt/PRY/3SR/2002/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/SEN/MRC-DPRU2051/2009/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/TGO/MRC-DPRU5123/2010/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/UGA/MSK-13-048/2013/G9P[8]	G9	P8	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/UGA/MUL-13-163/2013/G9P[8]	G9	P8	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/USA/2009727093/2009/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/USA/VU12-13-101/2013/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/USA/2008747307/2008/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/ZAF/MRC-DPRU2711/2008/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/ZWE/MRC-DPRU1102/2012/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/ZWE/MRC-DPRU1841/2009/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human/Bethesda/DC3/2009/G9P[8]	G9	P8	I1	R1	C1	M1	A1	N1	T1	X	H1
RVA/Human-wt/BEL/BE00045/2009/G1P[8]	G1	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BEL/BE00045/2009/G1P[8]	G1	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/UGA/MUL-13-157/2013/G1P[8]	G1	P8	I1	R1	C2	M1	A1	N1	T1	E1	H1
RVA/Human/Victoria/CK00015/2005/G1P[8]	G1	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/ESP/SS96217158/2015/G3P[8]	G3	P8	I2	R2	C2	M1	A2	N2	T2	E2	H2
RVA/Hu/RUS/Nov03-H54/2003/G4P[8]	G4	P8	I1	X							
RVA/Human-wt/Croatia/CR2006/2006/G8P[8]	G8	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/THA/SKT-98/2013/G1P[8]	G8	P8	I2	R2	C2	M1	A2	N2	T2	E2	H2
RVA/Hu-wt/ITA/ME659/14/2014/G12P[8]	G12	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Hu-wt/ITA/ME864/12/2012/G12P[8]	G12	P8	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BGD/Bang-068/2008/G9P[4]	G9	P4	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human/IND/RV09/2010/G9P[4]	G9	P4	I2	R2	C2	M2	X	N2	T1	X	H2
RVA/Human/IND/RV11/2010/G9P[4]	G9	P4	I2	R2	C2	M2	A2	N2	T2	E6	H2
RVA/Human/IND/RV10/2010/G9P[4]	G9	P4	I2	R2	X	M2	A2	N2	T2	E6	H2
RVA/Human-wt/IND/Kol-006/2011/G9P[4]	G9	P4	I2	R2	C2	M2	A2	N1	T2	E6	H2
RVA/Human-wt/IND/Kol-040/2012/G9P[4]	G9	P4	I2	R2	C2	M2	A2	N1	T2	E6	H2
RVA/Human-wt/ITA/AN18/2016/G9P[4]	G9	P4	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/JPN/S120088/2012/G9P[4]	G9	P4	I2	R2	C2	M2	A2	N2	T1	E2	H2
RVA/Human/KOR/Seoul1918/2012/G9P[4]	G9*	P4*	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/PRY/1157A/2007/G9P[4]	G9	P4	I2	R2	C2	M2	A2	N2	T1	E3	H2
RVA/Human-wt/USA/LB1562/2010/G9P[4]	G9	P4	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human/AUS/RCH041/2010/G2P[4]	G2	P4	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/AUS/CK20028/2006/G2P[4]	G2	P4	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BGN/J331/2010/G2P[4]	G2	P4	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/ESP/SS68949094/2015/G2P[4]	G2	P4	I2	R2	X						
RVA/Human-wt/JPN/CH1020/2016/G2P[4]	G2	P4	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/JPN/MI1132/2016/G2P[4]	G2	P4	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/USA/CNMC123/2011/G2P[4]	G2	P4	I2	R2	C2	M2	A2	N1	T2	E2	H2
RVA/Hu/RUS/O1117/2011/G2P[4]	G2	P4*	I2	X							

*Figure 7: Comparison of the Lebanese G9P[8] and G9P[4] viruses with selected human strains; Lebanese specimens are in bold; green shaded area indicates Wa-like origin; red shaded area indicates DS-1 like origin; yellow shaded area indicates unique genotype; X indicates no data available; * indicates partial genome*

C. Phylogenetic Analysis and Sequence Identity

VP7

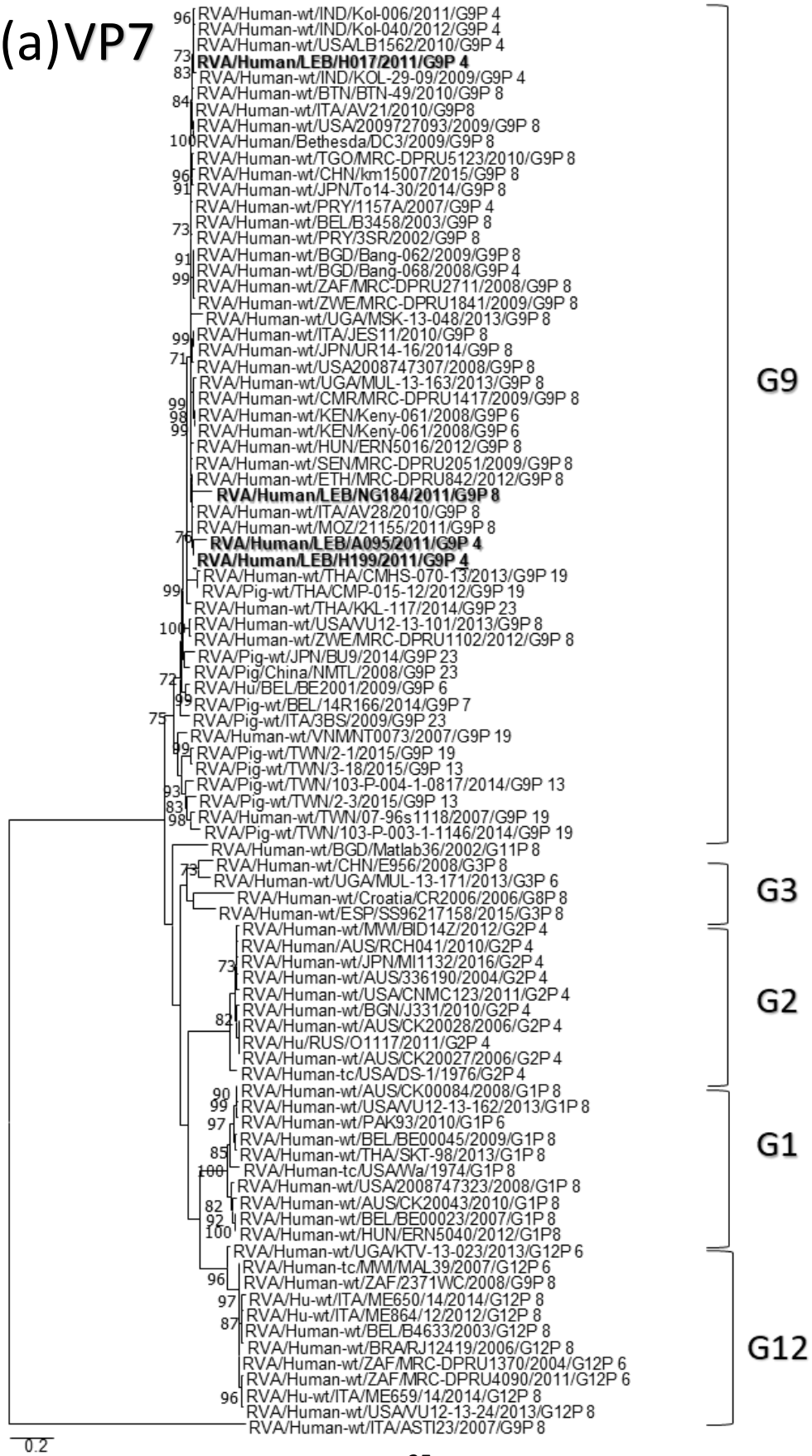
Based on the VP7 phylogenetic tree (Figure 8a-8a.1), all Lebanese strains clustered within the G9 lineage as expected. The NG184, A095 and H199 viruses clustered closely within the G9 lineage (90.9% to 93.9% nucleotide sequence identity) as opposed to the H017, which distantly located to these specimens (90.5% to 98.0%) despite belonging to the same lineage. H017 G9P[4] was found in a separate sub-lineage and was closely related to the Indian KOL-29-09 G9P[4], American LB1562 G9P[4] viruses (98.3% to 99.8%) as well as G9P[8] viruses from China, Japan, Belgium, and Bangladesh.

NG184 G9P[8] and the A095 and H199 G9P[4] specimens were most closely related to the Ethiopian MRC-DPRU842 G9P[8] (91.4% , 92.9%, 98.9%), the Italian AV28 G9P[8] (90.9%, 92.8%, 98.7%) and the Mozambique 21155 G9P[8] viruses (91%, 93.5%, 99.5%). Interestingly, these three human Lebanese specimens shared common ancestry with porcine G9P[19] and G9P[23] strains that were identified in Thailand, Japan, and China; Japanese BU9 G9P[23] (86.2% to 92.1% sequence identity), Chinese NMTL G9P[23] (87.4% to 93.8% sequence identity), Belgian 14R166 G9P[7] (86.7% to 92.8% sequence identity) and Italian 3BS G9P[23] (86.4% to 91.7% sequence identity). This suggests a potential interspecies transmission of the G9 strains between humans and pigs. The VP7 tree analysis also highlights the common ancestry of the of the VP7 gene of the G9P[4] and G9P[8] strains.

VP4

The VP4 phylogenetic tree (Figure 8b) classified the NG184 G9P[8] specimen into the expected P[8] lineage and A095, H017 and H199 G9P[4] into the P[4] lineage. NG184 G9P[8] shared highest similarity with Ethiopian MRC-DPRU842 G9P[8] virus (99.5% sequence identity), Senegal MRC-DPRU2051 G9P[8] and Jordanian J597 G1P[8]strains (99% to 99.4%), as well as Belgian and South African G9P[8] strains.

H199 and A095 G9P[4] are very closely related to each other on the phylogenetic tree (98.9% nucleotide identity). Both Lebanese specimens clustered near Australian RCH041 G2P[4] virus (97.2% to 99.7% identity), Japanese S120088 G9P[4] (98.2% to 98.3%) and Paraguayan 1157A G9P[4] (98.5% to 98.7%) specimens. H017 G9P[4] was most closely related to American LB1562 G9P[4] strain (99.3% sequence identity) and clustered near Bangladesh Bang-068 G9P[4] and J331 G2P[4] samples (99% to 99.4% sequence identity).


(a)VP7
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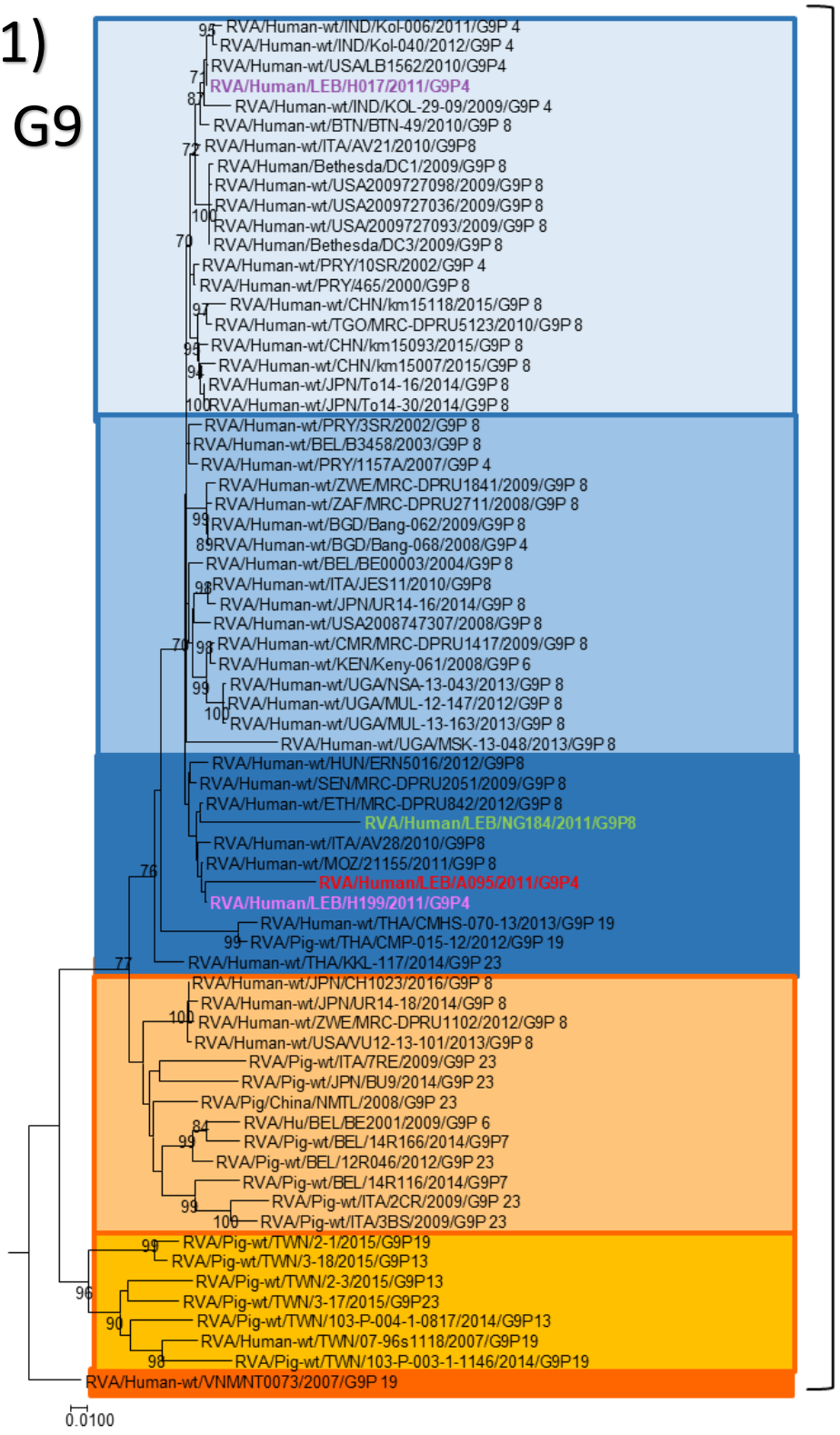
G3
G2
G1
G12
0.2

(a.1)
VP7 G9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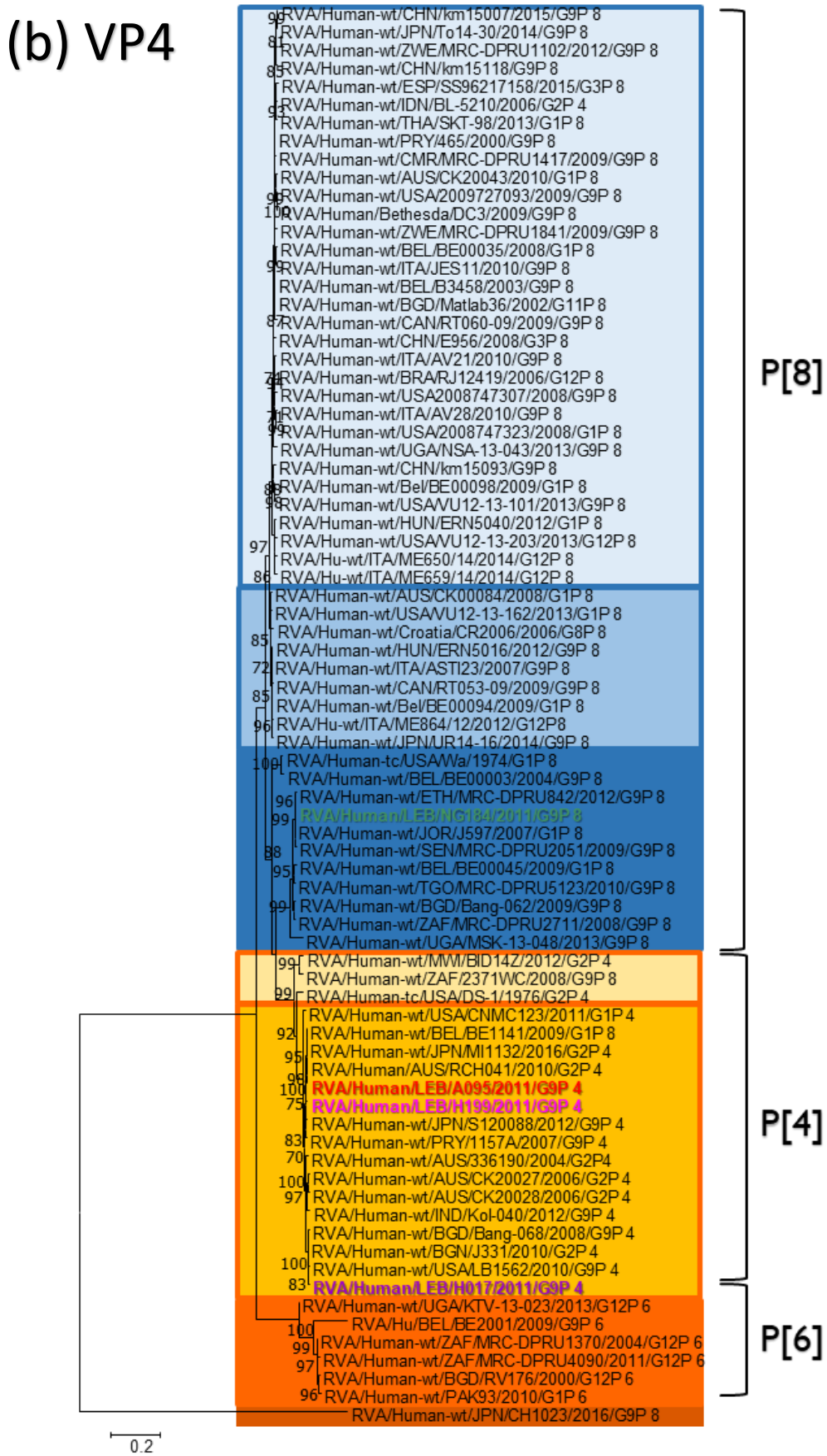
G9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P[8]
P[4]
P[6]
0.2

Figure 8: Phylogenetic analysis of full-length nucleotide sequences of the VP7 (a), VP7-zoomed in G9 lineage (a-1), VP4 (b), NSP1 (c), NSP2 (d), NSP3 (e), NSP4 (f), NSP5 (g), VP1(h), VP2(i), VP3 (j), VP6 (k) genes of human Lebanese specimens with other globally circulating RVA strains. The tree was generated by using the maximum-likelihood method in MEGA V8.0 and a bootstrap value of 1000. Bold font represents the Lebanese study specimens. Blue shapes and font represents strains of Wa-like origin and orange shapes and fonts represent strains of DS-1-like origin. Green bold font represents Lebanese NG184 strain, pink bold font represents Lebanese H199 strain, Purple bold font represents Lebanese H017 strain and red bold font represents Lebanese A095 strain. Blue gradient boxes indicate different sublineages of Wa-like origin. Orange gradient boxes indicate different sublineages of DS-1 like origin. For convenience purposes, VP7 and VP4 genotypes are colored based on different sublineages and non-Lebanese singletons are not assigned sublineages.

NSP1

The NSP1 gene (Figure 8c) of the NG184 G9P[8] clustered within the A1 genotype and was distantly related to the other Lebanese specimens (81.2% to 89.5%). A095 and H017 G9P[4] clustered in the two sublineages A2.2/ A2.3 (97.3%), whereas H199 G9P[4] was a singleton designated sublineage A2.1 (85.0% to 86.0% sequence identities). Although, NG184 G9P[8] showed highest nucleotide sequence identity with Italian JES11 G9P[8] and Ethiopian MRCU-DPRU842 G9P[8] (92% to 92.5%), they did not display a significant evolutionary relationship, as they clustered far from each other.

Interestingly, H199 G9P[4], which belonged to A2, showed high nucleotide sequence identities with the majority of strains from A1, such as Lebanese NG184 G9P[8] (89.5%) , Italian AV28 G9P[8] and Hungarian ERN5016 G9P[8] viruses (86.9%). Samples that showed comparable nucleotide sequence identities and belonged to the same A2 genotype as H199 include Pakistani PAK93 G1P[6], Australian CK20027/28 G2P[4], and Bangladesh J331 G2P[4] (shared 86.2% sequence identities). A095 G9P[4] exhibited maximum similarity with Australian RCH041 G2P[4] strain (99.7%) and fell on the same branch as American CNMC123 G2P[4] and Japanese MI1132 G2P[4] viruses (98.4% to 99.1%). H017 G9P[4] was most closely related to Indian Kol-040 G9P[4] and American LB1562 G9P[4] samples (99.1% to 99.5% sequence identities). A095

and H017 also clustered near other Bangladesh, Australian, Pakistan, American and Spanish viruses.

NSP2

The NSP2 genes (Figure 8d) of H199 G9P[4], H017 G9P[4] and NG184 G9P[8] (89.9% to 99.1% sequence identities) clustered within two different sublineages in the N1 genotype, whereas A095 G9P[4] clustered separately within the N2 genotype. H199 G9P[4] was found to exhibit maximum similarity with Chinese E956 G3P[8] strain (99.6% sequence identity) as well as Chinese km15093 G9P[8] and Zimbabwe MRC-DPRU1102 G9P[8] strains (98.7% to 99.3%). Additionally, it clustered near Belgian, Italian, Japanese and American viruses. H017 G9P[4] was most closely related to Belgian BE00045 G1P[8] specimen (99.5%) and fell on the same branch as Korean Seoul0373 G9P[8], Korean Seoul0918 G9P[4] and Italian ME659 G12P[8] strains (98.3% to 98.5% sequence identities). As such, H199 and H017 G9P[4] viruses clustered in two different clades of the same N1.1 sublineage, which clustered distantly from NG184 G9P[8] in sublineage N1.2. NG184 was identical to Ethiopian MRC-DPRU842 G9P[8] strain and was related to RVA/Human-wt/Croatia/CR2006/2006/G9P8 and Senegal MRC-DPRU2051 G9P[8] viruses (99.1% to 99.6% sequence identities).

A095 G9P[4] specimen shared ancestry with Australian RCH041 G2P[4] and Japanese MI1132 G2P[4] viruses (99.3% to 99.7%) and was related to a variety of globally circulating strains from countries such as Bangladesh, Uganda, China and Paraguay.

NSP3

The NSP3 gene (Figure 8e) of NG184 G9P[8] and H199 G9P[4] clustered in the sublineages T1.2 and T1.3 of the T1 genotype (94.8% sequence identity). On the contrary, although A095 and H017 G9P[4] viruses clustered in sublineages T2.2 and T2.3 they were more related to each other (96.6% identity) than NG184 and H199. Although NG184 G9P[8] shared high nucleotide sequence identity with Italian ME864 G12P[8], Ethiopian MRC-DPRU842 G9P[8] and Belgian BE00094 G1P[8] strains (94.7% to 95.4% sequence identities), they are found distantly from each other on the phylogenetic tree. Likewise, H199 G9P[4] showed high nucleotide sequence identities with Brazilian RJ12419 G12P[8], American 2008747323 G1P[8], and Belgian BE00045 G1P[8] viruses (94.1% to 94.7% sequence identities), however they did not display a significant evolutionary relationship.

A095 G9P[4] exhibited maximum similarity with Australian RCH041 G2P[4] (99.8%) virus and fell on the same branch as Japanese MI1132 G2P[4] (99.6%). H017 G9P[4] specimen shared ancestry with Bangladesh J331 G2P[4], Indian Kol-040, and American LB1562 G9P[4] viruses (99.1%, 99.2%, 99.3% sequence identities) and clustered near other Bangladesh, American and Malawian samples.

NSP4

The NSP4 gene (Figure 8f) of NG184 G9P[8] and H199 G9P[4], clustered in the same clade of the same sublineage in E1 (95.6% sequence identity), whereas A095 and H017 G9P[4]clustered in two different sublineages of E2 (91.6% sequence identity). NG184 G9P[8] specimen was most closely related to Ethiopian MRC-DPRU842 G9P[8] (99.5%) and fell on the same branch as Belgian BE0003 G9P[8] virus (99.0%). H199 G9P[4] similarly clustered near these specimens, in

addition to Belgian BE00094, Italian AV21 and ASTI23 G9P[8] and Croatian CR2006 G8P[8] (98.6% to 99.3% sequence identities).

A095 G9P[4] shared ancestry with RVA/Human-wt/MWI/BID14Z/2012/G2P4 (99.0%), as well as Australian RCH041and Japanese MI1132 G2P[4] specimens (99.0% to 98.9%). H017 G9P[4] was most closely related to Spanish SS96217158 G3P[8], Australian CK20028/27 G2P[4] viruses (98.7% to 99.2%). These Lebanese specimens also clustered near Pakistan, Bangladesh and Malawian samples.

NSP5

In the phylogenetic tree of the NSP5 gene (Figure 8g), NG184 G9P[8] and H199 G9P[4] clustered in the two different sublineages of H1 (95% sequence identity). This was similar to the different sublineages of A095 and H017 G9P[4] specimens in the H2 genotype (98.6% sequence identity). NG184 G9P[8] was identical to Ethiopian MRC-DPRU842 G9P[8] virus and shared ancestry with samples from Zimbabwe MRC-DPRU1841 G9P[8], Italy ME659 G12P[8], Hungary ERN5040 G1P[8] and South Africa MRC-DPRU4090 G12P[6] (97.8% to 98.3% sequence identities). Furthermore, NG184 clustered near global strains from Korea, Brazil, USA among others. H199 G9P[4] specimen was most closely related to Italian AV28 G9P[8] (98%) and American strains 2008747323 G1P[8] and VU12-13-162 G1P[8] (97.3%). This sample also clustered near Croatian, Australian, Canadian and Hungarian strains.

A095 G9P[4] was identical to Australian RCH041 G2P[4] specimen and they were most closely related to Japanese MI1132 G2P[4] and Malawian BID14Z G2P[4] strains (98.6% to 98.8% sequence identities). H017 G9P[4] was identical to Australian CK20027/28 strains and shared ancestry with another Australian 336190 G2P[4] virus (99.1%). A095 and H017 G9P[4] shared

ancestry with similar strains, such as Malawian MAL39 G12P[6], USA CNMC123 G2P[4], Spanish SS96217158 G3P[8], Pakistani PAK93 G1P[6] and some Bangladesh viruses.

VP1

VP1 gene (Figure 8h) of H199 G9P[4] and NG184 G9P[8] specimens were closely related (98.5%) belonging to the same sublineage R1.3. These were located further away from H017 G9P[4] and A095 G9P[4] which belonged to two distinct sublineages in the R2 genotype (94.5% sequence identity). H199 and NG184 shared ancestry with Ethiopian MRC-DPRU842 G9P[4] (98.5% to 98.8% sequence identities) and clustered near globally circulating viruses such as Italian ME864 G12P[8], Belgian BE00003 G9P[8], Senegal MRC-DPRU2051 G9P[8] (97.7% to 97.9%). H017 G9P[4] was most closely related to American LB1562 G9P[4] and Indian Kol-040 G9P[4] strains, as well as Bangladesh J331 G2P[4] viruses (99.3% to 99.6% sequence identities). A095 G9P[4] shared ancestry with Australian RCH041 and Japanese MI1132 G2P[4] viruses (99.1% to 99.7%). Furthermore, A095 clustered near Malawian, Uganda and Paraguayan samples.

VP2

In the phylogenetic tree of VP2 (Figure 8i), H199 G9P[4] and NG184 G9P[8] viruses fell in the same C1.2 sublineage (97.9%). These specimens shared 77.4% to 78.4% sequence identity with H017 and A095 G9P[4] samples. H017 and A095 G9P[4] belonged to C.2.3 sublineage and shared 98.3% sequence identity. NG184 G9P[8] and H199 G9P[4] were most closely related to Ethiopian MRC-DPRU842 G9P[8] virus (96.9% to 97.1 %). Furthermore, these clustered near Victorian CK00015 G1P[8] and Italian AV28 G9P[8] strains (96.6% to 97.1%). Other specimens in the same lineage originated from USA, Croatia, Hungary and some South Africa.

H017 G9P[4] specimen was most closely related to Pakistan PAK93 G1P[6] sample (99.3%), and shared a common ancestor with American CNMC123 G2P[4] strain (99% sequence identity).

A095 G9P[4] was most closely related to Australian RCHO41 G2P[4] virus (99.8%) and clustered near Malawian BID14Z and Japanese MI1132 G2P[4] samples (98.1% to 99.3% sequence identities). H017 and A095 also clustered near viruses from Bangladesh, Uganda, Paraguay and Spain.

VP3

The VP3 gene (Figure 8j) of H199 G9P[4] and NG184 G9P[8] specimens located in separate clusters of the same sublineage M1.2 (95% sequence identity). H017 and A095 G9P[4] specimens belonged to M2.1 and M2.3 sublineages (78.8% sequence identity), respectively. H199 G9P[4] virus shared ancestry with RVA/Human-wt/SEN/MRC-DPRU2051/2009/G9P8, Belgian BE0003 G9P[8] and Croatian CR2006 G8P[8] (94.6% to 96% sequence identities). NG184 G9P[8] was most closely related to Ethiopian MRC-DPRU842 G9P[8] specimen (98.2%) and clustered near Italian ME864 G12P[8] and ASTI23 G9P[8], Belgian BE00094 G1P[8] and Canadian RT053-09 G9P[8] (96.6% to 96.7% sequence identities).

H017 G9P[4] on the phylogenetic tree seemed to be identical to Australian CK20028/20027 G2P[4] strains (99.2% sequence identity) and shared a common ancestor with Pakistani PAK93 G1P[6] and American CNMC123 G2P[4] specimens (97.6% to 99.6%). These viruses also clustered near some Bangladesh, Indian and other American viruses. A095 G9P[4] shared ancestry with Australian RCH041 G2P[4] and Japanese MI1132 G2P[4] viruses (99.3% to 99.6%). Furthermore, they clustered near globally circulating strains from Bangladesh, Uganda, Malawian, Spain and Paraguay.

VP6

The VP6 gene (Figure 8k) of NG184 G9P[8] and H199 G9P[4] viruses clustered in different sublineages (87.9%) in the I1 genotype. H199 and A095 G9P[4] were located in two I2.2 and I2.3

sublineages (97.2%). NG184 G9P[8] specimen shared highest similarity with Ethiopian MRC-DPRU842 G9P[8] virus (98.3%) and clustered near USA 2008747323 and Thailand SKT-98 G1P[8] specimens (98.0% to 98.1% sequence identities), as well as other samples from Belgian, Italy and Croatia. Although H199 G9P[4] shared 87.8% sequence identity with American 2008747323 G1P[8] and Ethiopian MRC-DPRU842 G9P[8] samples, as well as 87.7% with Thailand SKT-98 G1P[8] and 87.6% with Italian AV28 G9P[8] specimens, they did not display a significant evolutionary relationship.

H017 G9P[4] shared ancestry with Thailand SKT-457 G8P[8], Australian CK20028/CK20027 G2P[4], Belgian J331G2P[4] and Russian O1117 G2P[4] viruses (87.7% to 99.4% sequence identities) and clustered near Indian Kol-40 and American LB1562 G9P[4] viruses. A095 G9P[4] was most similar to Australian RCH041 (99.8% sequence identity) and shared a common ancestor with Malawian BID14Z and Japanese MI1132 G2P[4] viruses (98.8% to 99.1%). Furthermore,

these specimens clustered near Paraguayan, Spanish, Bangladesh, South African, Uganda and Australian viruses

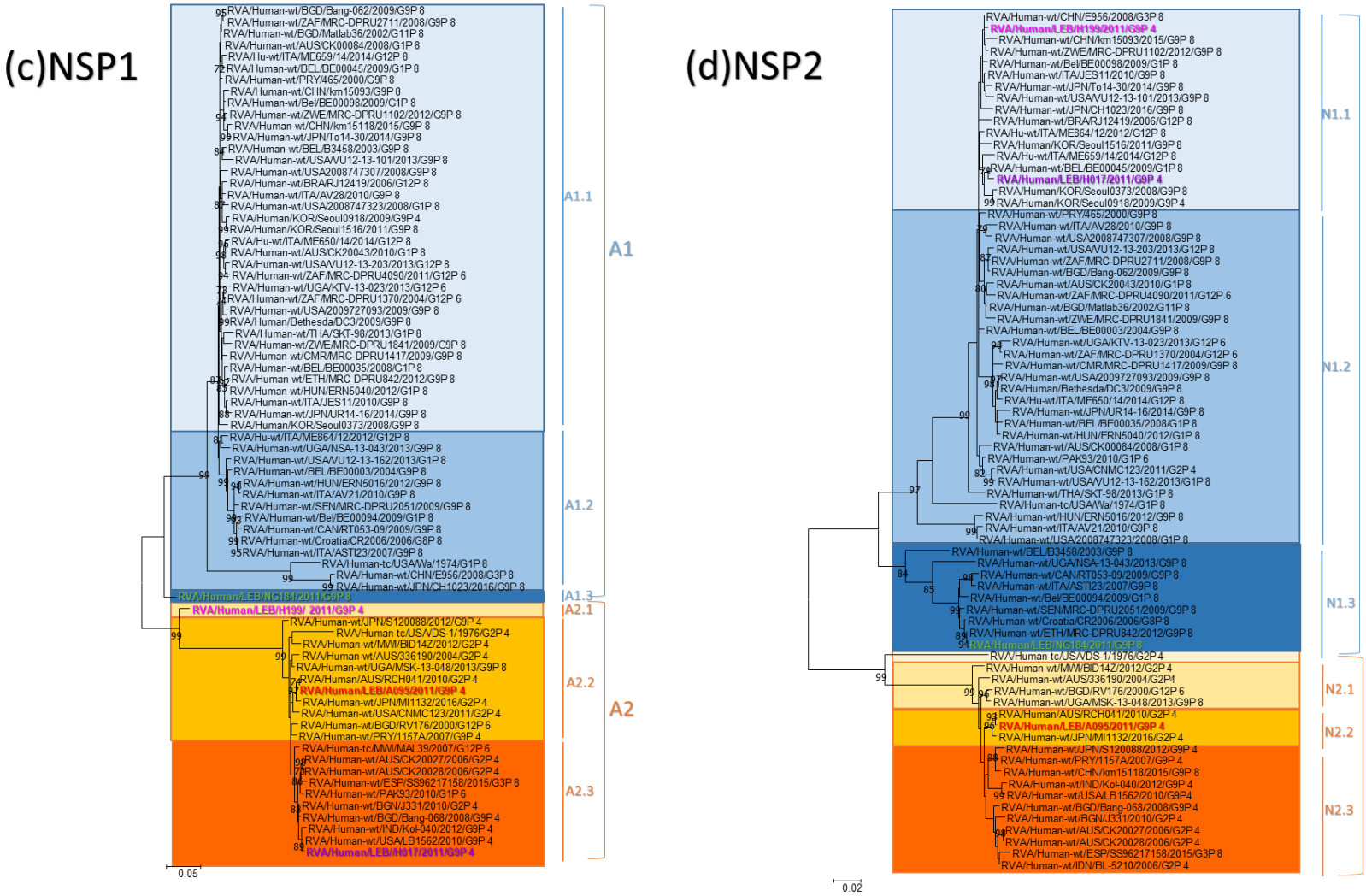

Figure 8 (continued)

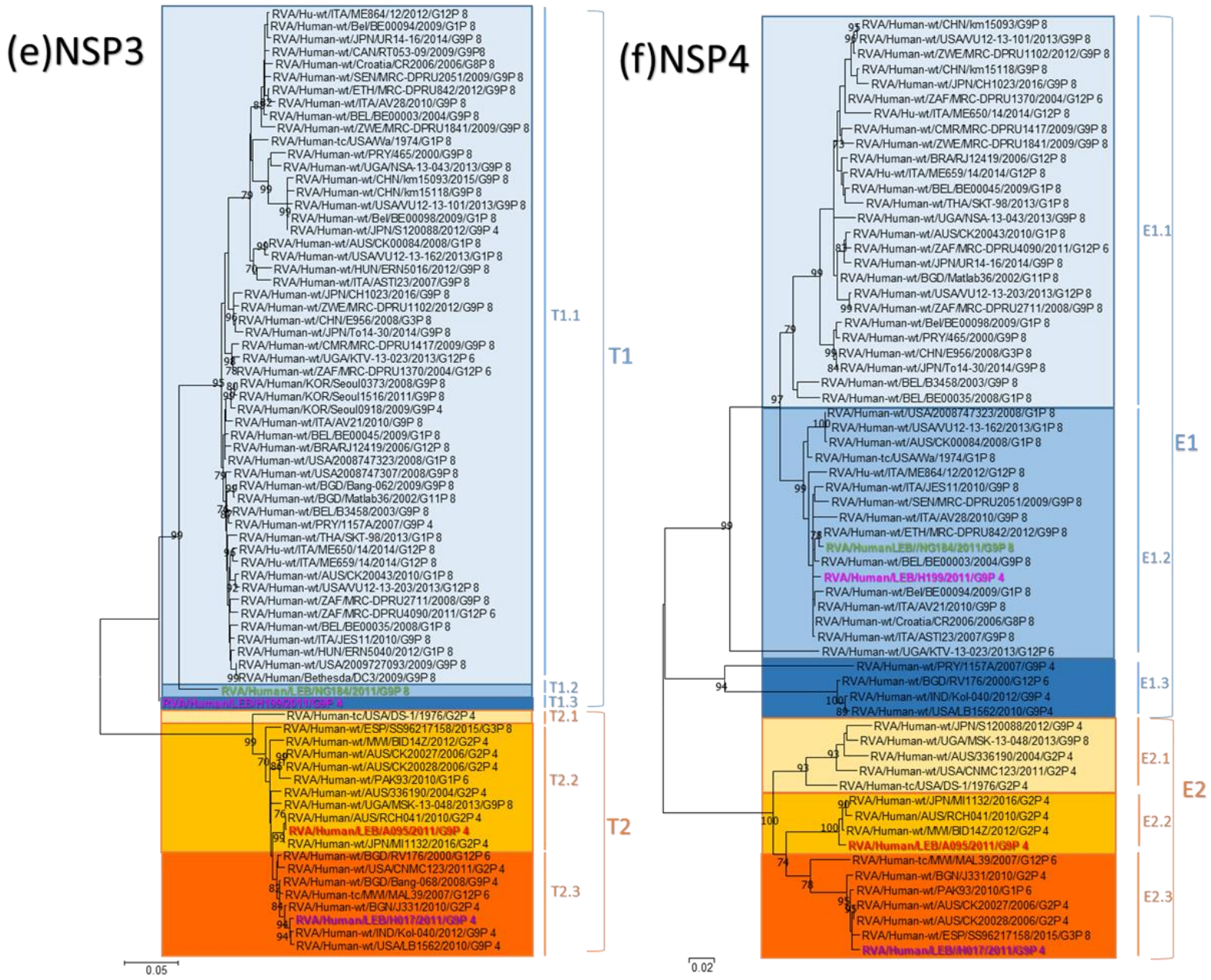

Figure 8 (continued)

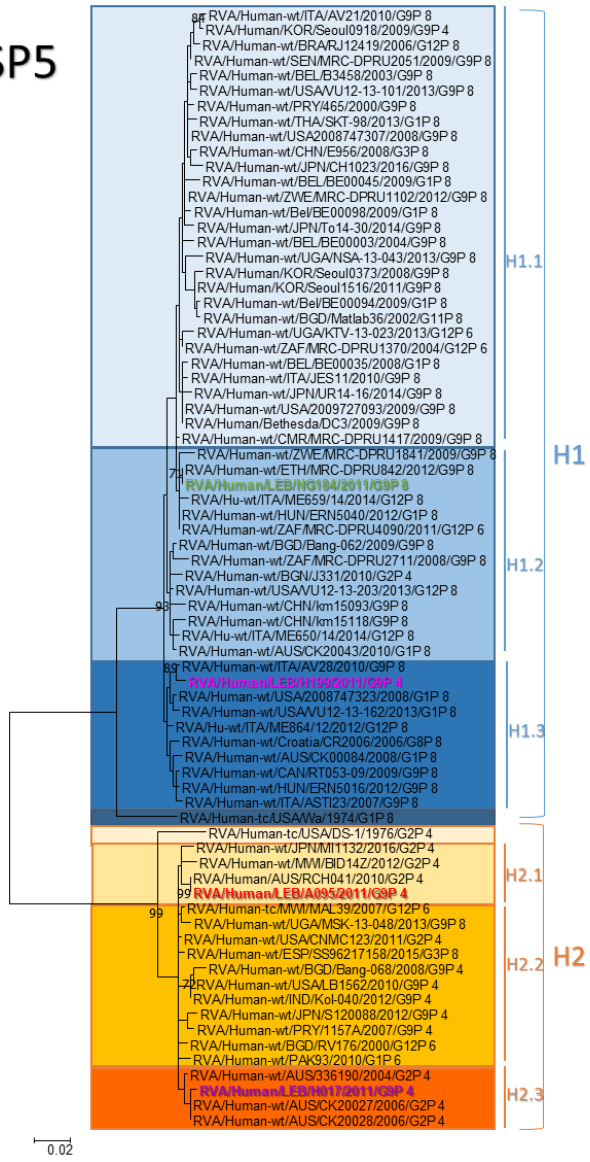

Figure 8 (continued)

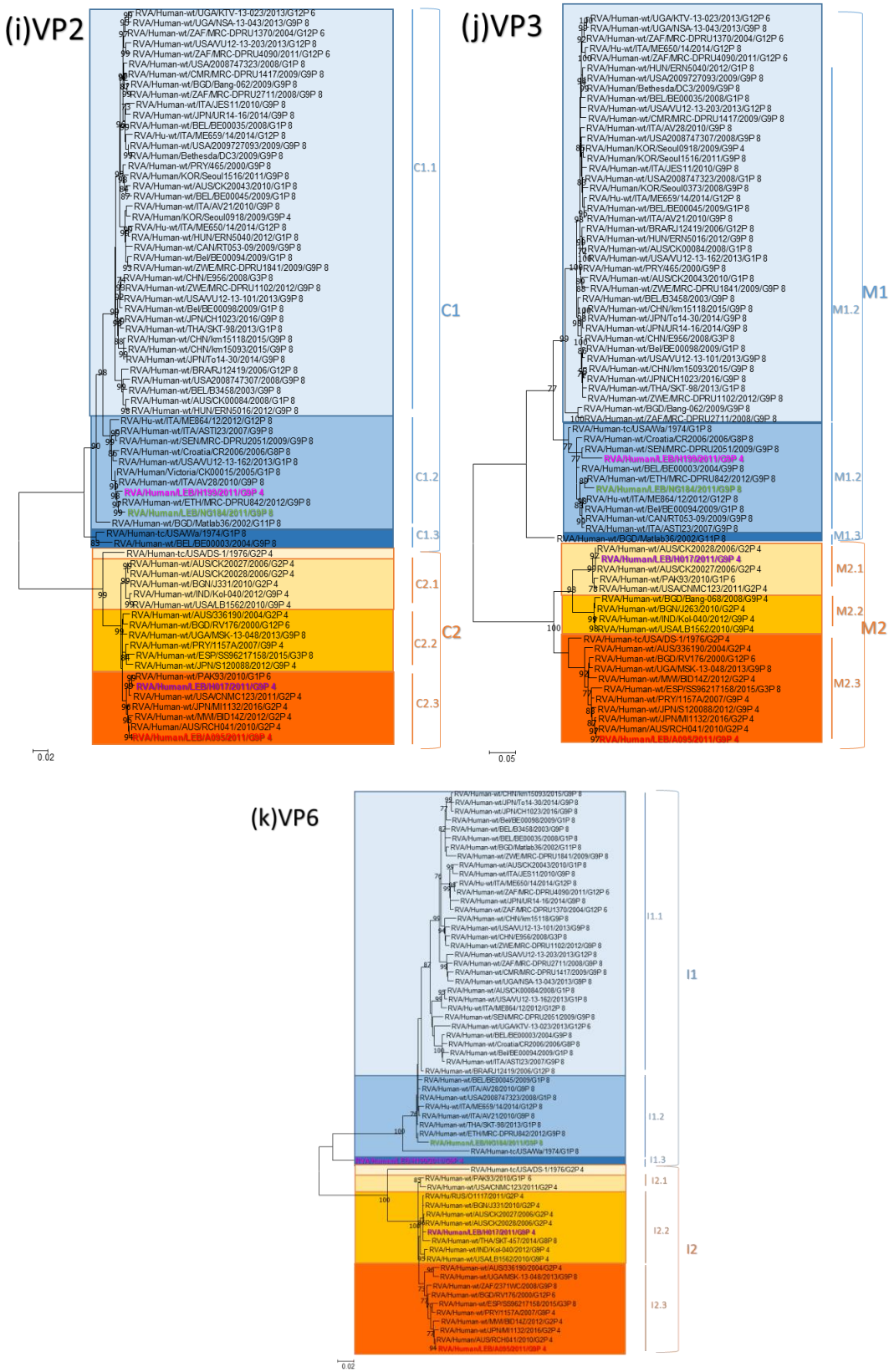

Figure 8 (continued)

D. Phylogenetic analysis summary

The phylogenetic tree analysis was summarized to provide an eagle eye view of the different genomic constellations and relationships identified among the various strains (Figure 9). The strains can be divided into 3 groups: DS-1 like origin, Wa-like origin and mixed genotypes. Interestingly, aside from the Lebanese A095 strain which had a pure DS-1 like genotype, all globally circulating and representative G9P[4] specimens had a mixed genotype with a largely DS-1 like backbone similar to G2P[4] strains. A095 had identical genomic constellation with Japanese MI1132 G2P[4] strains suggesting a common ancestry of these strains. H199 was the sole G9P[4] with a mainly Wa-like backbone and was more similar to G9P[8] than G9P[4] strains. Nearly all the reported G9P[8] strains including NG184 possessed a pure Wa-like constellation, although intragenotypic reassortment events were evident. NG184 had identical genomic constellation in 9 segments with the Ethiopian MRC-DPRU842 strain, providing clues to the potential origin of these strains. Only one G9P[8] strain from Uruguay (MSK-13-084) possessed a pure DS-1 like constellation that resembled G2P[4] strains, suggesting a reassortment event between these two genotypes.

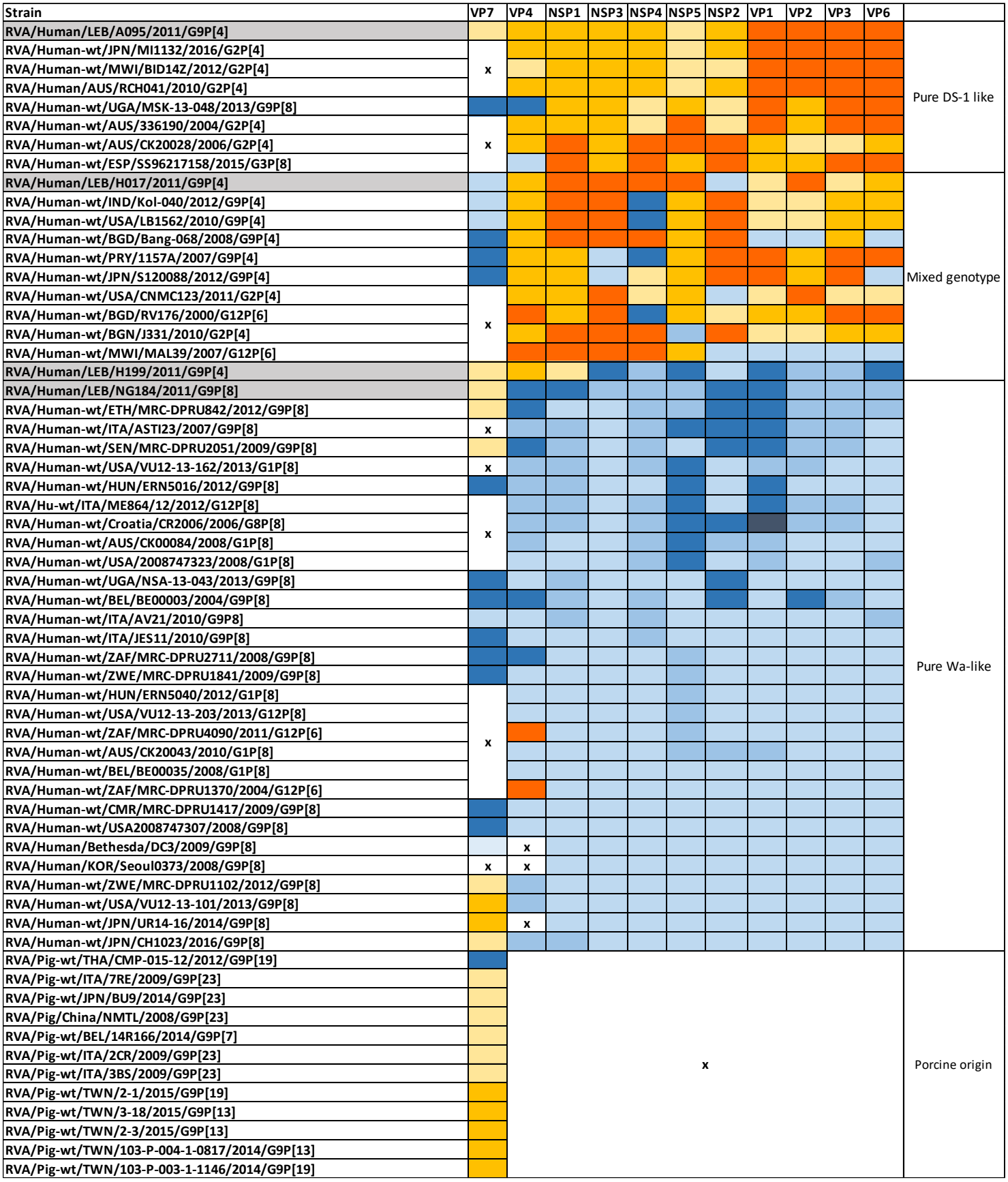
Strain
VP7
VP4
NSP1
NSP3
NSP4
NSP5
NSP2
VP1
VP2
VP3
VP6
RVA/Human/LEB/A095/2011/G9P[4]
RVA/Human-wt/JPN/MI1132/2016/G2P[4]
RVA/Human-wt/MWI/BID14Z/2012/G2P[4]
RVA/Human/AUS/RCH041/2010/G2P[4]
RVA/Human-wt/UGA/MSK-13-048/2013/G9P[8]
RVA/Human-wt/AUS/336190/2004/G2P[4]
RVA/Human-wt/AUS/CK20028/2006/G2P[4]
RVA/Human-wt/ESP/SS96217158/2015/G3P[8]
Pure DS-1 like
RVA/Human/LEB/H017/2011/G9P[4]
RVA/Human-wt/IND/Kol-040/2012/G9P[4]
RVA/Human-wt/USA/LB1562/2010/G9P[4]
RVA/Human-wt/BGD/Bang-068/2008/G9P[4]
RVA/Human-wt/PRY/1157A/2007/G9P[4]
RVA/Human-wt/JPN/S120088/2012/G9P[4]
RVA/Human-wt/USA/CNMC123/2011/G2P[4]
RVA/Human-wt/BGD/RV176/2000/G12P[6]
RVA/Human-wt/BGN/J331/2010/G2P[4]
RVA/Human-wt/MWI/MAL39/2007/G12P[6]
Mixed genotype
RVA/Human/LEB/H199/2011/G9P[4]
RVA/Human/LEB/NG184/2011/G9P[8]
RVA/Human-wt/ETH/MRC-DPRU842/2012/G9P[8]
RVA/Human-wt/ITA/ASTI23/2007/G9P[8]
RVA/Human-wt/SEN/MRC-DPRU2051/2009/G9P[8]
RVA/Human-wt/USA/VU12-13-162/2013/G1P[8]
RVA/Human-wt/HUN/ERN5016/2012/G9P[8]
RVA/Hu-wt/ITA/ME864/12/2012/G12P[8]
RVA/Human-wt/Croatia/CR2006/2006/G8P[8]
RVA/Human-wt/AUS/CK00084/2008/G1P[8]
RVA/Human-wt/USA/2008747323/2008/G1P[8]
RVA/Human-wt/UGA/NSA-13-043/2013/G9P[8]
RVA/Human-wt/BEL/BE00003/2004/G9P[8]
RVA/Human-wt/ITA/AV21/2010/G9P8]
RVA/Human-wt/ITA/JES11/2010/G9P[8]
RVA/Human-wt/ZAF/MRC-DPRU2711/2008/G9P[8]
RVA/Human-wt/ZWE/MRC-DPRU1841/2009/G9P[8]
RVA/Human-wt/HUN/ERN5040/2012/G1P[8]
RVA/Human-wt/USA/VU12-13-203/2013/G12P[8]
RVA/Human-wt/ZAF/MRC-DPRU4090/2011/G12P[6]
RVA/Human-wt/AUS/CK20043/2010/G1P[8]
RVA/Human-wt/BEL/BE00035/2008/G1P[8]
RVA/Human-wt/ZAF/MRC-DPRU1370/2004/G12P[6]
RVA/Human-wt/CMR/MRC-DPRU1417/2009/G9P[8]
RVA/Human-wt/USA2008747307/2008/G9P[8]
RVA/Human/Bethesda/DC3/2009/G9P[8]
RVA/Human/KOR/Seoul0373/2008/G9P[8]
RVA/Human-wt/ZWE/MRC-DPRU1102/2012/G9P[8]
RVA/Human-wt/USA/VU12-13-101/2013/G9P[8]
RVA/Human-wt/JPN/UR14-16/2014/G9P[8]
RVA/Human-wt/JPN/CH1023/2016/G9P[8]
Pure Wa-like
RVA/Pig-wt/THA/CMP-015-12/2012/G9P[19]
RVA/Pig-wt/ITA/7RE/2009/G9P[23]
RVA/Pig-wt/JPN/BU9/2014/G9P[23]
RVA/Pig/China/NMTL/2008/G9P[23]
RVA/Pig-wt/BEL/14R166/2014/G9P[7]
RVA/Pig-wt/ITA/2CR/2009/G9P[23]
RVA/Pig-wt/ITA/3BS/2009/G9P[23]
RVA/Pig-wt/TWN/2-1/2015/G9P[19]
RVA/Pig-wt/TWN/3-18/2015/G9P[13]
RVA/Pig-wt/TWN/2-3/2015/G9P[13]
RVA/Pig-wt/TWN/103-P-004-1-0817/2014/G9P[13]
RVA/Pig-wt/TWN/103-P-003-1-1146/2014/G9P[19]
x
Porcine origin

Figure 9: Summary of the phylogenetic trees. VP7 refers to the G9 lineage only. The Lebanese specimens are shaded in gray. The Lebanese specimens are shaded in gray. Different shades of blue indicate RVA strains of Wa-like origin. Different shades of orange indicate RVA strains of DS-1 like origin. X indicates strains not available in the phylogenetic tree of the particular genotype

E. Amino acid sequence identity between Lebanese rotavirus A strains and vaccine viruses

To understand the genetic relatedness between Lebanese strains and Rotarix and RotaTeq vaccines, a similarity matrix based on the amino acid constitution of the VP7 and VP4 genes was inferred (Figure 10). The G9 VP7 of 3/4 Lebanese specimens shared highest amino acid sequence identity with G3 VP7 of RotaTeq (80.1% to 83.3%) whereas the NG184 virus shared 81.2% sequence identity with G2 VP7 of RotaTeq. As for the VP4 genotype, the highest amino acid sequence identity in the G9P[4] Lebanese specimens was shared with the P[8] VP4 of RotaTeq (90.4% to 90.5%). VP4 of NG184 G9P[8] had high sequence P[8] VP4 of both Rotarix and Rotateq. We next compared the antigenic epitopes of the VP4 and VP7 in order to gain more in-depth understanding of the genetic vaccine-matching of the Lebanese G9P[4] and G9P[8] specimens.

Strain	Rotarix G1	Rotateq G1	Rotateq G2	Rotateq G3	Rotateq G4	Rotarix P[8]	Rotateq P[8]
RVA/Human/LEB/A095/2011/G9P[4]	77.00%	77.10%	79.10%	80.10%	73.80%	89.00%	90.40%
RVA/Human/LEB/H017/2011/G9P[4]	79.10%	78.00%	76.10%	83.30%	76.50%	89.30%	90.40%
RVA/Human/LEB/H199/2011/G9P[4]	79.10%	78.00%	76.10%	83.30%	76.20%	89.10%	90.50%
RVA/Human/LEB/NG184/2011/G9P[8]	78.50%	77.40%	81.20%	80.40%	75.00%	92.20%	91.90%

Figure 10: Distance matrix for VP7 and VP4 based on amino acid identities between RotaTeq and Rotarix vaccines and Lebanese specimens. Green shaded area indicates highest amino acid sequence identity of the VP7 genotype. Red shaded area indicates highest amino acid sequence identity of the VP4 genotype

F. VP7 antigenic epitopes of the Lebanese RVA strains and vaccine viruses

Epitope 7-1a

Analysis of the VP7 antigenic epitopes revealed that the Lebanese G9 specimens possessed a similar composition of the 7-1a epitope, except for the S87T substitution of A095 (Figure 11).

Compared to the G1 components of the RotaTeq and the Rotarix vaccines, the Lebanese samples had 5-6 amino acid substitutions at positions 87,94, 96,123,125,129 of 7-1a antigenic epitope that are different from both vaccines. An additional amino acid substitution at residue 97 was identified in our specimens compared to G1 VP7 of RotaTeq.

Compared to the G2 strain of RotaTeq, our specimens differed at positions 87,91,94,96 99, 100,125,129 and 130 of VP7.

Compared to G3 VP7 of RotaTeq, one can observe that all Lebanese specimens had shared substitutions at residues 94, 96, 97, and 129. A095 G9P[4] possessed an additional unique substitution at residue 87.

Finally, comparing the G4 specimen of RotaTeq, the Lebanese strains possessed four substitutions at residues 87, 94, 123, and 125, except for A095 which lacked the change at residue 87.

Epitope 7-1b

In contrast to the 7-1a epitope, this epitope showed more similarities in the amino acid constitution between A095, H017 and H199 viruses, whereas the NG184 virus had more distinctive amino acid residues. Nevertheless, there was a conserved N242T substitution in all Lebanese samples.

Compared to the G1 of RotaTeq and Rotarix vaccine strains, A095, H017 and H199 viruses possessed similar substitutions at residues 212 and 213. While NG184 possessed an amino acid change at residue 211.

Compared to the G2 VP7 of RotaTeq, the G9P[4] strains had only one (1/6) residue in epitope 7-1b that was similar to the vaccine strain. While NG184 G9P[8] had changes in 3/6 residues.

Compared to G3 VP7 of RotaTeq, the majority (4-5/6) of 7-1b residues were found to be mutated in the Lebanese strains.

Compared to the G4 VP7 of RotaTeq, the A095, H017 and H199 G9P[4] strains had 2 and 3 substitutions in this epitope, one being common among all strains (N242 T)

Epitope 7-2

Compared to the G1 strains of RotaTeq and Rotarix vaccines, only positions 145 and 264 were conserved. All Lebanese viruses differed at positions 146, 147, 217 and 221 from both vaccines. Additionally, NG184 possessed substitutions at positions 143, 148 and 190.

Compared to the G2 VP7 of RotaTeq, A095, H017 and H199 differed at positions 143, 146, 148,190 and 217. Interestingly, NG184 virus had an identical 7-2 epitope to G2 of RotaTeq.

Compared to the G3 strain of RotaTeq, the Lebanese specimens shared a common substitution at residue 221. Additionally, the G9P[4] specimens possessed a change at residue 146. NG184 had substitutions at positions 143, 146, 148, 190 and 217.

Compared to G4 of RotaTeq, the Lebanese specimens shared two common substitutions at residues 145 and 147. A095, H017 and H199 had additional mutations at residues 143, 148, and 217; whereas NG184 possessed mutations at residues 146,148,190 and 217.

In conclusion, the Lebanese G9P4] specimens were more similar antigenically to G4 VP7 of RotaTeq vaccine, given the least number of mutations observed compared to this strain.

	Epitope 7-1a														Epitope 7-1b						Epitope 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
G1 A41CB052A/1988/G1P1A[8]/RotaRix	T	T	N	G	E	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	N	L	S	M	N	G
G1 WI79-9/1992/G1P7[5]/RotaTeq	T	T	N	G	D	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	S	L	S	M	N	G
A095 G9P[4]	S	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
H017 G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
H199 G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
NG184 G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	D	V	D	D	N	R	D	N	T	S	D	I	S	G
G2 SC2-9/P[5]/1981/RotaTeq	A	N	S	D	E	W	E	N	Q	D	T	M	N	K	Q	D	V	S	N	S	R	D	N	T	S	D	I	S	G
A095 G9P[4]	S	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
H017 G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
H199 G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
NG184 G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	D	V	D	D	N	R	D	N	T	S	D	I	S	G
G3 WI78-8/1992/G3P7[5]/RotaTeq	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
A095 G9P[4]	S	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
H017 G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
H199 G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
NG184 G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	D	V	D	D	N	R	D	N	T	S	D	I	S	G
G4 BrB/P[5]/1984/RotaTeq	S	T	S	T	E	W	K	D	Q	N	L	I	D	K	Q	D	T	A	D	T	R	A	S	G	E	S	T	S	G
A095 G9P[4]	S	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
H017 G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
H199 G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
NG184 G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	D	V	D	D	N	R	D	N	T	S	D	I	S	G

Figure 11: Alignment of antigenic residues in VP7 between the strains contained in Rotarix and RotaTeq and Lebanese specimen. Antigenic residues are divided into three epitopes (7-1a, 7-1b, and 7-2). Red font color indicates amino acids of Rotarix strains. Blue font color indicates amino acids of RotaTeq strains. Yellow shaded residues are residues that differ between Rotarix and RotaTeq strain. Red residues are amino acids that differ between the Lebanese specimens and both Rotarix and RotaTeq. Blue shaded residues are amino acids that differ between the Lebanese specimens and RotaTeq.

G. VP4 antigenic epitopes of circulating Lebanese RVA strains and vaccine viruses

VP8*

Analysis of the VP8* epitope residues of all G9P[4] Lebanese specimens revealed that they have identical amino acid constitutions, which differed from the G9P[8] Lebanese specimens (Figure 12A).

In the 8-1 epitope, Lebanese specimens possessed a similar composition at positions 100,150,190,193 and 194. Compared to the P[8] RotaTeq and Rotarix vaccines, all Lebanese viruses had S150N and D193N changes. Interestingly, NG184 G9P[8] varied in all positions except 100 and 194 from both vaccines. Furthermore, A095, H017 and H199 G9P[4] differed from P[8] of RotaTeq at position 196 and from P[8] of Rotarix at position 190.

In the 8-2 epitope, only NG184 G9P[8] had a G183N substitution that was different from both vaccines.

In the 8-3 epitope, all Lebanese samples and vaccine strains had a conserved residue at position 132. Additionally, A095, H017 and H199 G9P[4] were different from both vaccines at positions 113 – 116, 131 and 133. Moreover, these 3 samples differed from P[8] of Rotarix at residues 125 and 135. NG184 G9P[8] differed from both vaccines at position 113, from RotaTeq virus at positions 125 and 135 and from Rotarix at position 135.

In the 8-4 epitope, only G9P[4] specimens possessed a change in residue 89.

VP5*

Analysis of the VP5* epitope residues, showed that all 4 Lebanese specimens differed only at positions 388, 393 and 306 (Figure 12B).

In the 5-1 epitope, all specimens were different from RotaTeq at position 384 and from both vaccines at position 386. A095, H017 and H199 G9P[4] samples experienced mutations at positions 388 and 393 that were different from the mutation in NG184 (S388T).

In the epitope 5-5, only A095, H017 and H199 G9P[4] samples possessed a mutation in residue 306.

In conclusion, we can conclude that the Lebanese P[4] specimens were antigenically more similar to VP4 P[8] of RotaTeq since they experienced less mutations compared to Rotarix, whereas the P[8] Lebanese specimen was more similar to VP4 P[8] of Rotarix.

A)

	8-1											8-2		8-3									8-4		
	100	146	148	150	188	190	192	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89
P[8] A41CB052A/G1/1988/Rotarix	D	S	S	N	S	S	A	N	L	N	N	E	R	N	P	V	D	S	S	N	D	N	N	T	N
P[8] W179-4/G1/1983/RotaTeq	D	S	S	N	S	N	A	N	L	N	D	E	R	N	P	V	D	N	R	N	D	D	N	T	N
A095 G9P[4]	D	S	S	S	S	N	A	D	L	N	N	E	R	S	Q	T	N	N	E	N	S	D	N	T	D
H017 G9P[4]	D	S	S	S	S	N	A	D	L	N	N	E	R	S	Q	T	N	N	E	N	S	D	N	T	D
H199 G9P[4]	D	S	S	S	S	N	A	D	L	N	N	E	R	S	Q	T	N	N	E	N	S	D	N	T	D
NG184 G9P[8]	D	N	I	S	G	N	S	D	L	I	S	E	G	D	P	V	D	S	R	N	D	N	N	T	N

B)

	5-1								5-2	5-3	5-4	5-5
	384	386	388	393	394	398	440	441	434	459	429	306
P[8] A41CB052A/G1/1988/Rotarix	S	Y	S	A	W	N	L	R	E	N	S	L
P[8] W179-4/G1/1983/RotaTeq	R	H	S	A	W	N	L	R	E	N	S	L
A095 G9P[4]	S	D	R	E	W	N	L	R	E	N	S	S
H017 G9P[4]	S	D	R	E	W	N	L	R	E	N	S	S
H199 G9P[4]	S	D	R	E	W	N	L	R	E	N	S	S
NG184 G9P[8]	S	D	T	A	W	N	L	R	E	N	S	L

Figure 12: Alignment of antigenic residues in VP4 between the strains contained in Rotarix and RotaTeq and Lebanese specimens. In panel A the antigenic residues are divided in three antigenic epitopes in VP8. In panel B antigenic residues are divided in five antigenic epitopes in VP5*. Red font color indicates amino acids of Rotarix strain. Blue font color indicates amino acids of RotaTeq strains. Yellow shaded residues are amino acids that differ between Rotarix and RotaTeq strains. Purple shaded residues are amino acids that differ between sample strains and both Rotarix and RotaTeq strains. Blue shaded residues are amino acids that differ between sample strains and RotaTeq strain*

H. Comparison of the antigenic epitopes among the globally circulating G9P[8] and G9P[4] rotaviruses

In order to understand the significance of the mutations in the epitopic residues of the Lebanese specimens, we compared their antigenic epitopes with representative G9P[8] and G9P[4] strains. A consensus sequence was constructed based on the most representative amino acid residue at each position. Overall, the VP7 epitopes were largely conserved among G9P[4] and G9[8] strains (Figure 13). Most of the study viruses contain one or less mutations in these epitopes. Surprisingly, in the Lebanese NG184 G9P[8] strain, numerous mutations compared to the rest of the G9 strains were observed. In case of the VP4 antigenic epitopes, as expected, a significant number of substitutions were observed among the G9P[4] compared to the G9P[8] (Figure 14). Yes, the majority of the VP4 antigenic residues were conserved among viruses of the same genotype. The conservation of the antigenic epitopes of the VP7 gene of G9 strains provide an opportunity for the development of a vaccine strain that can target these emerging strains.

	Epitope 7-1a														Epitope 7-1b						Epitope 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
Consensus	**T**	**T**	**G**	**T**	**E**	**W**	**K**	**D**	**Q**	**D**	**A**	**I**	**D**	**K**	**Q**	**N**	**T**	**A**	**D**	**N**	**K**	**D**	**S**	**T**	**L**	**S**	**E**	**S**	**G**
RVA/Human-wt/IND/Kol-006/2011/G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	N	G
RVA/Human-wt/BGD/Bang-068/2008/G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	S	K	D	S	T	L	S	E	S	G
RVA/Human-wt/IND/Kol-040/2012/G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/PRY/1157A/2007/G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/USA/LB1562/2010/G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human/LEB/A095/2011/G9P[4]	S	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human/LEB/H017/2011/G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human/LEB/H199/2011/G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human/LEB/NG184/2011/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	D	V	D	D	N	R	D	N	T	S	D	I	S	G
RVA/Human-wt/CHN/km15007/2015/G9P[8]	T	T	G	A	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/TGO/MRC-DPRU5123/2010/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	G	G
RVA/Human-wt/ITA/JES11/2010/G9P[8]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/USA/VU12-13-101/2013/G9P[8]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/USA2008747307/2008/G9P[8]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/JPN/UR14-16/2014/G9P[8]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/ZWE/MRC-DPRU1102/2012/G9P[8]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/ZWE/MRC-DPRU1841/2009/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	S	K	D	S	T	L	S	E	S	G
RVA/Human-wt/BGD/Bang-062/2009/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	S	K	D	S	T	L	S	E	S	G
RVA/Human-wt/ZAF/MRC-DPRU2711/2008/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	S	K	D	S	T	L	S	E	S	G
RVA/Human-wt/BEL/B3458/2003/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/ETH/MRC-DPRU842/2012/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/ITA/ASTI23/2007/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/ITA/AV21/2010/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/ITA/AV28/2010/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human/Bethesda/DC3/2009/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/JPN/To14-30/2014/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/MOZ/21155/2011/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/PRY/3SR/2002/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/SEN/MRC-DPRU2051/2009/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/UGA/MSK-13-048/2013/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/UGA/MUL-13-163/2013/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RVA/Human-wt/USA/2009727093/2009/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G

Figure 13: Alignment of antigenic residues in VP7 between reference strains and Lebanese specimens. Antigenic residues are divided in three antigenic eptiopes (7-1a,7-1b,7-2). Consensus was constructed based on the most representative amino acid residues. Yellow shading indicates difference among available rotavirus strains and consensus

	VP8*																										VP4*																									
	8-1											8-2		8-3										8-4			5-1									5-2		5-3		5-4		5-5	
	100	146	148	150	188	190	192	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89	384	386	388	393	394	398	440	441	434	459	429	306													
Consensus	**D**	**S**	**S**	**S**	**S**	**N**	**A**	**N**	**L**	**N**	**G**	**E**	**R**	**D**	**P**	**V**	**D**	**N**	**R**	**N**	**D**	**D**	**N**	**T**	**N**	**S**	**D**	**S**	**A**	**W**	**N**	**L**	**R**	**E**	**N**	**S**	**L**													
RVA/Human-wt/IND/Kol-040/2012/G9P[4]	D	S	S	S	S	N	A	D	L	N	N	E	R	S	P	T	N	N	E	N	S	D	N	T	D	S	D	R	E	W	N	L	R	E	N	S	S												
RVA/Human-wt/JPN/S120088/2012/G9P[4]	D	S	S	S	S	N	A	D	L	N	N	E	R	S	Q	T	N	N	E	N	S	D	N	T	D	S	D	R	E	W	N	L	R	E	N	S	S												
RVA/Human-wt/PRY/10SR/2002/G9P[4]	D	S	S	S	S	N	A	D	L	N	N	E	R	S	Q	T	N	N	E	N	S	D	N	T	D	S	D	R	E	W	N	L	R	E	N	S	S												
RVA/Human-wt/PRY/1157A/2007/G9P[4]	D	S	S	S	S	N	S	D	L	N	N	E	R	S	Q	T	N	N	E	N	S	D	N	T	D	S	D	R	E	W	N	L	R	E	N	S	S												
RVA/Human-wt/USA/LB1562/2010/G9P4	D	S	S	S	S	N	A	D	L	N	N	E	R	S	Q	T	N	N	E	N	S	D	N	T	D	S	D	R	E	W	N	L	R	E	N	S	S												
RVA/Human/LEB/A095/2011/G9P4	D	S	S	S	S	N	A	D	L	N	N	E	R	S	Q	T	N	N	E	N	S	D	N	T	D	S	D	R	E	W	N	L	R	E	N	S	S												
RVA/Human/LEB/H017/2011/G9P4	D	S	S	S	S	N	A	D	L	N	N	E	R	S	Q	T	N	N	E	N	S	D	N	T	D	S	D	R	E	W	N	L	R	E	N	S	S												
RVA/Human/LEB/H199/2011/G9P4	D	S	S	S	S	N	A	D	L	N	N	E	R	S	Q	T	N	N	E	N	S	D	N	T	D	S	D	R	E	W	N	L	R	E	N	S	S												
RVA/Human/LEB/NG184/2011/G9P8	D	N	I	S	G	N	S	D	L	I	S	E	G	D	P	V	D	S	R	N	D	N	N	T	N	S	D	T	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/ETH/MRC-DPRU842/2012/G9P[8]	D	N	N	S	G	N	S	D	L	I	S	E	G	D	P	V	D	S	R	N	D	N	N	T	N	S	D	T	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/SEN/MRC-DPRU2051/2009/G9P[8]	D	N	S	S	G	N	S	D	L	I	S	E	G	D	P	V	D	S	R	N	D	N	N	T	N	S	D	T	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/TGO/MRC-DPRU5123/2010/G9P[8]	D	S	N	S	G	N	A	D	L	T	S	E	G	D	P	V	D	S	R	N	D	N	N	T	N	S	D	T	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/ZAF/MRC-DPRU2711/2008/G9P[8]	D	S	S	S	G	N	A	D	L	T	S	E	G	D	P	V	N	S	W	N	D	N	N	T	N	S	D	T	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/BEL/B3458/2003/G9P[8]	D	S	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/ITA/ASTI23/2007/G9P8	D	S	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/ITA/AV21/2010/G9P8	D	S	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/ITA/AV28/2010/G9P8	D	S	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	A	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/ITA/JES11/2010/G9P8	D	S	S	N	S	S	A	N	L	N	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/JPN/UR14-16/2014/G9P[8]	D	S	S	N	S	N	A	N	L	D	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/USA/VU12-13-101/2013/G9P[8]	D	S	S	N	S	N	T	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/USA2008747307/2008/G9P[8]	D	S	S	N	S	N	S	N	L	N	G	E	R	D	P	A	D	N	R	N	D	D	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/ZWE/MRC-DPRU1841/2009/G9P[8]	D	S	S	N	S	N	A	N	L	N	G	E	R	N	P	V	D	N	R	N	D	D	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												
RVA/Human/Bethesda/DC3/2009/G9P[8]	D	S	S	N	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/ZWE/MRC-DPRU1102/2012/G9P[8]	D	S	S	S	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/JPN/To14-30/2014/G9P[8]	D	S	S	S	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												
RVA/Human-wt/CHN/km15007/2015/G9P[8]	D	S	S	S	S	N	A	N	L	N	G	E	R	D	P	V	D	N	R	N	D	D	N	T	N	S	D	S	A	W	N	L	R	E	N	S	L												

Figure 14: Alignment of antigenic residues in VP4 between reference strains and Lebanese specimens. Antigenic residues are divided in three antigenic epitopes in VP8. Antigenic residues are divided in five antigenic epitopes in VP4*. Consensus was constructed based on the most representative amino acid residues. Yellow shading indicates difference among available rotavirus strains and consensus.*

CHAPTER 5:
DISCUSSION

Rotavirus is considered the major cause of gastroenteritis in children under the age of 5 years and is associated with a significant mortality and morbidity in developing and developed countries (WHO, 2013). Vaccination is currently the mainstay for prevention and is effective at reducing the burden of rotavirus as countries who introduced the vaccine saw a marked drop in rotavirus-associated severe gastroenteritis (WHO, 2009). Currently available Rotarix and RotaTeq vaccines are 85% to 98% effective against severe rotavirus illnesses and associated hospitalizations, and 74% to 87% against rotavirus illnesses of any severity in children under 1 year of age (Cortese & Parashar, 2009). Nevertheless, vaccine efficacy in low-income countries, such as in Africa and Asia, seems to be lower, which could be attributed to factors such as poor nutrition, enteric coinfections, acquiring natural rotavirus infection in young infancy, and interference of maternal antibodies with vaccine response (Madhi et al, 2012; Madhi et al, 2016; Tissera et al, 2017). Vaccine efficacy could be affected by possible immune selection. The wide use of rotavirus vaccines could drive antigenic drifts or shifts of circulating strains towards otherwise less common genotypes, which may not be effectively covered by the available vaccines (Dennehy et al, 2008). For instance, recent studies including one from Lebanon demonstrated an increased prevalence of G9P[8] genotype and the emergence of G9P[4] genotype. This raises concern on the long term efficacy of current RV vaccines and highlights the need for characterizing

these emerging genotypes (Doan et al, 2017; Yamamoto et al, 2015; Yarkin et al, 2016; Chieochansin et al, 2016; Ali et al, 2016).

In this study, we analyzed the full genomes of three G9P[4] and one G9P[8] rotavirus A strains that were identified in Lebanon from 2011-2013.Interestingly, our G9P[4] viruses were not identical at the genomic level. As such, two out of the three G9P[4] viruses possessed a mixed Wa-like/DS-1 like genotype. In contrast to G9P[8] strains which generally possessed a pure Wa-like constellation, the G9P[4] strains from Lebanon and other countries possessed a mixed genomic backbone that was very diverse. In case of G9P[8] strains, intragenotypic reassortments seem to happen more frequently than intergenotypic reassortments.

The emergence of the G9P[4] strain is hypothesized to be the result of reassortment events between the commonly circulating G2P[4] and G9P[6] rotavirus strains or more recently between G2P[4] and the more prevalent G9P[8] viruses (Rahman et al, 2008; Chitambar et al, 2014). Consistently, Lebanese A095 and H017 G9P[4] strains possessed a genomic constellation that was highly similar to G2P[4] rotavirus strains reported from Australia, Japan, Bangladesh and Malawi (Southeastern Africa), while their VP7 was very similar to G9P[8] strains detected in Lebanon and other countries. Similarly, H199 G9P[4] seemed to originate from a reassortment event between G2P[4] and G9P[8] strains, whereby the majority of genes were acquired from the G9P[8] backbone. This supports the suggestion that recent G9P[4] strains are a product of a reassortment event between G2P[4] and G9P[8] strains. Other G9P[4] strains possessing non-DS-1 or Wa-like genes were reported in the United States, Japan, India, and Latin America (Lewis et al, 2014; Yamamoto et al, 2015; Doan et al, 2017; Ruiz-Palacios et al, 2006). This suggests that more than one lineage of G9P[4] has emerged in Lebanon and other countries through independent

reassortment and introduction events. Rotavirus genes tend to remain in unique sets that offer a balanced fitness for the virus (McDonald et al, 2009). The high genomic diversity of the G9P[4] strain suggests that this genotype possesses a flexible genome that has a tendency to accept segments from various backgrounds. Another explanation for this observation is that this genotype is still at its early emergence stage and thus has not yet acquired a stable and fit genomic composition that enables its spread efficiently worldwide as a stable lineage. Furthermore, the fact that the Lebanese strains did not share an identical genomic constellation with global strains could suggest possible de novo emergence of these unique genotypes. Nevertheless, the lack of data on full genome analysis of G9P[4] strains in Lebanon and globally, curtail a definitive conclusion on the source of these rotavirus strains.

The observed reassortment events were all linked to rotavirus strains of human origin emphasizing the importance of human-to-human transmission in the emergence of new strains and their introduction to other countries. The Lebanese NG184 strain shared recent ancestry with another G9P[8] strain (MRC-DPRU842) from Ethiopia. A large population of Ethiopians work in Lebanon as house helps and nannies, which could suggest a potential transmission route for the introduction of novel rotavirus genotypes into these countries.

Furthermore, the VP7 genotype of NG184 G9P[8], H199 G9P[4], and A095 G9P[4] were accommodated in the same cluster as G9P[19] strains of human and porcine origin identified in Thailand (Yodmeeklin et al, 2017). Studies including ours reveal that human G9P[8] possess similar Wa-like genomic constellation as porcine viruses further supporting the role of zoonotic transmission of rotavirus in the emergence of human strains (Matthijnssens et al, 2008).

The emergence of unique and diverse rotavirus genotypes was observed after rotavirus vaccine introduction possibly due to vaccine pressure (Markkula et al, 2017). Nevertheless,

Lebanon did not introduce rotavirus vaccines into their national immunization program and the vaccination rate is relatively low (18.1%) (Ali et al, 2016). As such, the emergence of these unique genotypes could not be solely explained by vaccine pressure locally but rather a global trend that led to the introduction of these new genotypes to Lebanon.

One of G9P[4] specimens in this study originated from a child who was previously vaccinated with the RotaTeq vaccine, but still presented with severe gastroenteritis. While our data remains limited to one case, it might indicate suboptimal protection by the vaccine against this genotype. The VP7 genes of G9P[4] rotaviruses shared highest similarity with VP7 G3 of RotaTeq (80.1% to 83.3%) and with VP4 P[8] of RotaTeq (90.4% to 90.5%). This was in contrast to the Lebanese G9P[8] specimen which shared highest similarity with VP7 G2 of RotaTeq (81.2%) and P[8] of Rotarix (92.2%) (Figure 14). Mutations in the epitopic residues of VP7 and VP4 might influence vaccine efficacy since they play an important role in the binding ability of neutralization antibodies (Estes and Kapikian, 2007; Morozova et al, 2015; Fredj et al, 2013; Ruggeri and Greenberg, 1991; Zhou et al, 1994). In our study, only 34.5% to 62.1% of the epitopic residues of the G9P[4] specimens' VP7 were conserved compared to vaccine G1-G4 strains. In case of the G9P[8] specimen 48.3% to 55.2 % of these residues were similar to the vaccine strain. Regarding the G9P[4] and G9P[8] VP4 epitopes, only 51.3% and 48.6% of the epitopes were conserved, respectively, compared to the P[8] of the vaccines. The globally circulating G2P[4] rotavirus strain has been shown to be associated with rotavirus vaccine breakthrough infections and outbreaks in several countries (Luchs et al, 2017; Tanaka et al, 2017; Matthijnssens et al, 2014). The epitopic residues of the VP7 genotype of Belgian and Tunisian G2P[4] rotavirus strains (Zeller et al, 2011; Ben Hadj Fredj et al, 2013) share 31.04% of the substitutions with our Lebanese strains and 79.3% similarity with VP7 G2 RotaTeq vaccine strain. Whereas the eptiopic residues of the VP4 genotype

of G2P[4] rotavirus strains share 97.03% with our Lebanese strains and 48.6% similarity to vaccine VP4 P[8] RotaTeq. Thus, our G9P[4] rotavirus strains share only 24.5% and 59.4% similarity in the VP7 and VP4 protein, respectively, of G2P[4] RotaTeq vaccine strains, and are therefore likely to be associated with vaccine breakthrough as observed with patient H017.

We can conclude that heterotypic protection via the immunodominant VP6 protein is critical for maintaining the efficacy of current vaccines against G9 rotaviurs A strains (Ruiz-Palacios, 2006; Correia et al, 2010; Yen et al, 2011). However, it should be noted that as G9 strains continue to evolve, the efficacy of the existing vaccines might be undermined (Cheng Xu et al, 2018; Chan-It & Chanta, 2017). Given this evolution and genotypic diversity, further research is needed for fully understand the mechanisms underlying heterotypic protection and how to enhance heterotypic protection of rotavirus vaccines to ensure their s long-term efficacies. Nevertheless, on the short term, adding emerging genotypes such as G9, might aid in controlling rotavirus outbreaks. Such an approach has been already employed in India for instance, where ROTAVAC vaccine with a VP7 G9 component has been introduced into the Universal Immunization Program in 2015 (Bhandari et al, 2014). The development and introduction of a G9-based vaccine is feasible and efficacious due to the fact that the antigenic epitopes within VP7 are highly conserved among the Lebanese and globally representative G9 rotavirus strains.

Our study had a few limitations including the small sample size of the Lebanese G9P[4] specimens hindering significant comparison with other G9 rotavirus strains. However, this was inherent to the fact that the G9P[4] was rarely detected in Lebanon. Moreover, we compensated for the limited number of specimens by including globally reported G9P[4] and G9P[8] strains.

Our data highlight the importance of monitoring the emergence and spread as well as the genomic diversity of G9P[4] as well as other emerging genotypes in Lebanon and other countries

to ensure maintaining the efficacy of existing vaccines. Further studies are needed to better understand the efficacy of current vaccines against emerging genotypes including G9P[4] and G9P[8]. Serum neutralization studies of sera obtained from vaccinated children against these genotypes are also warranted.

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