# Phylogenetic and Evolutionary Analyses of the VP4 Gene of P[9] Rotaviruses

## P[9] Rotavirüslerinin VP4 Geninin Filogenetik ve Evrimsel Analizleri

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**ABSTRACT**

**Objective:** Rotavirus is one of the major causes of gastroenteritis in children under 5 years of age. It can evolve by reassortment, in which gene segments are exchanged between strains of different origins. In some rotavirus strains the P[9] component is an example of reassortment, in which the P[9] genotype is from feline species. A number of outbreaks associated with P[9] strains have been documented in several countries. However, details regarding the epidemiological relationships between the strains remains largely unknown. Therefore, in the present study, genetic characterization and evolutionary analyses were performed to gain insight into P[9] strains circulating in different parts of the world.

**Materials and Methods:** A total of 94 full- and partial-length VP4 gene sequences of P[9] strains were extracted from GenBank and phylogenetic trees were constructed by maximum likelihood method. Timeline of evolution was performed using the full-length nucleotide sequences of VP4 genes of P[9] strains using the Bayesian Markov Chain Monte Carlo method available in BEAST version 1.6.1.

**Results:** The VP4 gene of the P[9] strains could be divided into two lineages, with lineage I is further divided into five sub-lineages. All the P[9] strains characterized in this study shared a common ancestor that circulated in circa 1864 (95% HPD 1755–1941). In each lineage, the strains were not only from different countries, but also from different continents. These findings suggest that none of the lineages has a specific region of distribution, and although humans have had interactions with cats for thousands of years, the common ancestor of the VP4 gene of the current P[9] strains is relatively recent.

**Conclusion:** These findings suggest that P[9] rotaviruses can be divided into two lineages. None of the lineages and sub-lineages has a specific region of distribution, and the ancestor of the current P[9] strain is relatively recent.

**Keywords:** Rotavirus, genotype P[9], phylogenetic analysis, lineages, time-line of evolution.

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**ÖZET**

**Amaç:** Rotavirüs, 5 yaş altındaki çocuklarda gastroenteritin başlıca nedenlerinden biridir. Gen segmentlerinin farklı kökenlerden gelen suşlar arasında reasortmana uğramasıyla evrimleşebilmektedir. Bazı rotavirüs suşlarında, kedigillerden gelen P[9] genotipindeki P[9] komponenti reasortmana bir örnektir. Birkaç ülkede P[9] suşları ile ilişkili bir dizi salgın belgelenmiştir. Bununla birlikte, suşlar arasındaki epidemiyolojik ilişkilere ilişkin ayrıntılar büyük ölçüde bilinmemektedir. Bu nedenle, bu çalışmada, dünyanın farklı bölgelerinde dolaşan P[9] suşları hakkında fikir edinmek için genetik karakterizasyon ve evrimsel analizler yapılmıştır.

**Gereç ve Yöntemler:** P[9] suşlarına ait toplam 94 tam ve kısmi uzunluktaki VP4 gen dizisi GenBankasından alınmış ve filogenetik ağaçlar 'maximum likelihood' yöntemi ile oluşturulmuştur. Evrimsel zaman çizelgesi, P[9] suşlarının VP4 genlerinin tam uzunluktaki nükleotit dizileri kullanılarak 'BEAST versiyon 1.6.1.'de mevcut olan 'Bayesian Markov Chain Monte Carlo' yöntemi ile yapılmıştır.

**Bulgular:** P[9] suşlarının VP4 geni iki kökene (lineage) ayrılmakta ve köken I, beş alt-kökene ayrılmaktadır. Bu çalışmadaki tüm P[9] suşları, 1864 dolaylarında (95% HPD 1755–1941) dolaşan ortak bir atayı paylaşmaktadır. Her kökende, suşlar sadece farklı ülkelerden değil, aynı zamanda farklı kıtalardandı. Bu bulgular, kökenlerin hiçbirinin belirli bir dağılım bölgesine sahip olmadığını ve insanlar binlerce yıldır kedilerle etkileşime girmiş olsa da, mevcut P[9] suşlarının VP4 geninin ortak atasının nispeten yeni olduğunu göstermektedir.

**Sonuç:** Bu bulgular, P[9] rotavirüslerinin iki kökene bölünebileceğini düşündürmektedir. Kökenlerin ve alt kökenlerin hiçbirinin belirli bir dağılım bölgesi yoktur ve mevcut P[9] suşunun atası nispeten yenidir.

**Anahtar Sözcükler:** Rotavirüs, genotip P[9], filogenetik analiz, köken, evrimsel zaman çizelgesi

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## INTRODUCTION

Rotavirus is a major cause of gastroenteritis and is responsible for over 200,000 deaths annually in children under 5 years of age (1). Rotavirus has a double-stranded RNA genome divided into 11 segments, encoding six structural (VP1–VP4, VP6, and VP7) and six nonstructural proteins (NSP 1–6) (2). A binary classification system developed on the basis of two outer capsid proteins, VP7 and VP4, using G and P genotypes is used to identify different strains of group A rotavirus. To date 36 G genotypes and 51 P genotypes have been found (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg). Among the numerous G/P-genotype combinations, only a limited number are commonly found in human infections; these are G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8](3).

One of the mechanisms by which rotavirus evolves is reassortment, where gene segments are exchanged between strains of different origins. Among the known reassortment strains, G3P[9] is the least studied, but has become prominent, where the P[9] gene, often detected in associated with G3, is feline-like (4). The common G genotypes in combination with P[9] are G3, G6, G1, and G12 (5-11). The incidence of P[9] strains causing infection in humans is relatively low, at about 2.5% worldwide (12). Despite low incidence, P[9] strains were detected and were partly responsible for multiple outbreaks in several countries over the years (13-23). In Brazil and Ireland, around 10% and 18% of the patients infected with rotavirus had P[9] strains (21, 24). Such examples raise concerns that P[9] strains might become more virulent over time with multiple reassortments and thus have the potential to cause further outbreaks in other countries.

Based on limited studies, the evolutionary patterns of the human P[9] rotaviruses appear to be complex (23). Therefore, the present study was performed to determine the genetic relationships among the VP4 gene of P[9] strains circulating in different countries, and their evolutionary timelines.

## MATERIALS and METHODS

*Phylogenetic analyses*

A total of 94 full- and partial-length VP4 gene nucleotide sequences of P[9] strains were extracted from GenBank (Table 1). Phylogenetic analyses were conducted with the maximum likelihood method using MEGA X (25) after aligning the nucleotide sequences using CLUSTAL W (26). The branching patterns were evaluated based on a bootstrap analysis of 1,000 replicates. In all of the phylogenetic trees, lineages were designated based on significant bootstrap values of >70%. Several phylogenetic trees were constructed using full-length gene and partial-length genes of different lengths (Table 2).

*Nucleotide identity*

The nucleotide identities of the full- and partial-length P[9] genes of different lineages were calculated by online software (www.bioinformatics.org).

*Timeline and evolution*

Evolutionary analysis was performed using the full-length nucleotide sequences of VP4 genes. We inferred a maximum clade credibility phylogenetic tree using the Bayesian Markov Chain Monte Carlo method available in BEAST version 1.6.1 (27). The nucleotide sequences were analyzed by using a relaxed molecular clock (uncorrelated lognormal) and general time-reverse model (GTR+I+Γ model). The sequences were run for 60 million generations and sampled at every 3,000 steps. The end result was a sample size of 2,000 Bayesian trees, which was then verified for convergence by Tracer version 1.5.

## RESULTS

*Phylogenetic analyses*

In the phylogenetic tree constructed using 43 full-length gene sequences of the VP4 gene of the P[9] strains, two lineages could be identified – lineage I and lineage II (Figure 1). Lineage I was further divided into five sub-lineages – sub-lineage Ia, Ib, Ic, Id and Ie. Lineage Ia consisted of G3P[9], G1P[9], G3G4P[9], and G12P[9] strain from Paraguay, Japan, China, Thailand and Korea. Paraguayan strains were of genotypes G1P[9], G3P[9], G3G4P[9], and G12P[9]. Strains from other countries were of genotype G3P[9] (Table 3). Sub-lineage Ib consisted of strains from Italy, Australia, and Japan. All these strains were of genotype G3P[9]. Sub-lineage Ic consisted of G3P[9] and G6P[9] strains from Russia, Japan, Italy, Korea, Hungary, and Tunisia. Among these, strains from Russia, Korea, Hungary, and Italy were G3P[9], while strains from Tunisia and Japan were G6P[9]. Sub-lineage Id consisted of strains from the USA, and Italy; all were G3P[9]. Sub-lineage Ie consisted of two strains from Lebanon of G3P[9]. Lineage II was not divided into sub-lineages and consisted of strains from Paraguay, Brazil, Italy and Thailand. All of the strains in lineage II were of the G12P[9] combination.

**Table 1.** Accession numbers of the 94 rotavirus P[9] outer capsid protein VP4 genes used for phylogenetic analysis throughout the experiment. The accession numbers were retrieved from the National Center for Biotechnology Information.

| Names of rotavirus strains | Accession number | Names of rotavirus strains | Accession number |
|---|---|---|---|
| **AU-1** | D10970 | **R138** | KJ820894 |
| **FRV-1** | D10971 | **R135** | KJ820883 |
| **MP-CIVET66** | AB526248 | **R70** | KJ820872 |
| **02-92** | AB008289 | **RUS-Nov06-K10** | FJ435204 |
| **E2451** | JX946171 | **CU-B1263/KK** | KT007763 |
| **CAU14-1-262** | KR262152 | **CU-B1264/KK** | KT007764 |
| **RAC-DG5** | AB526246 | **CMH120** | DQ923798 |
| **L621** | EU708574 | **CMH134** | DQ923802 |
| **CU365-KK** | JN706511 | **KC814** | AJ311735 |
| **1259A** | KJ412822 | **0537** | JF805012 |
| **1701SR** | KJ412623 | **PA43** | JF793939 |
| **1256A** | KJ412885 | **PA27-GV1** | JF793937 |
| **1702SR** | KJ412909 | **Arg720** | EU513174 |
| **1257A** | KJ412800 | **Kor588** | EU513177 |
| **1709SR** | KJ412855 | **K12** | EU513176 |
| **PAI58** | GU296427 | **CP1030** | AB125855 |
| **Cat2a (AUS)** | EU708959 | **CC425** | AJ311734 |
| **Cat2b (JPN)** | D13403 | **Hun7** | AJ488141 |
| **Nov10-N507** | JQ289055 | **Hun8** | AJ488142 |
| **O1154** | JX027618 | **Hun6** | AJ488140 |
| **17237** | JX271004 | **Hun2** | AJ488137 |
| **RUS-Nov07-2253** | FJ435205 | **Hun3** | AJ488138 |
| **O211** | JX435054 | **RV10109** | JQ715633 |
| **O1180** | JX027619 | **R1320** | HM035520 |
| **Omsk08-442** | GU320751 | **Hun1** | AJ480136 |
| **CAU12-2-51** | KJ187602 | **M318** | AB008667 |
| **KF17** | JF421978 | **O264** | AB008665 |
| **BA222** | GU827409 | **PA151** | D14623 |
| **12US1134** | KF500522 | **Ro-6460** | X90736 |
| **PAH136** | GU296426 | **AU125** | D14614 |
| **ERN5162** | KJ919655 | **512B** | AB008669 |
| **ME848** | KR632621 | **BG1465** | KM590395 |
| **PE15776** | KF907295 | **BG11** | KM590385 |
| **T152** | AB077766 | **BG1840** | KM590383 |
| **PE18974** | KF907294 | **BG932** | KM590384 |
| **1471SR** | KJ412578 | **BG885** | KM590391 |
| **942A** | KJ412601 | **BG1328** | KM590386 |
| **1037A** | KJ412534 | **BG1452** | KM590392 |
| **1135A** | KJ412844 | **BG1511** | KM590388 |
| **R49** | KJ820839 | **BG1458** | KM590393 |
| **R55** | KJ820850 | **BG1506** | KM590397 |
| **R142** | KJ820850 | **BG1461** | KM590394 |
| **R47** | KJ820828 | **BG291** | KM590396 |
| **R57** | KJ820861 | **BG1271** | KM590389 |
| **FRV317** | LC328214.1 | **FRV384** | LC328215.1 |
| **PG05** | KC152916.1 | **N235** | MN029118.1 |
| **N566** | MN029129.1 | **Arg721** | EU513175.1 |

**Table 2.** The number of rotavirus P[9] strains and the length of nucleotide sequences used to construct 12 phylogenetic trees.

| No. of strains | Position of nucleotides | Length of nucleotide sequences |
|---|---|---|
| **43** | 1–2328 | 2,328 |
| **51** | 16–2328 | 2,313 |
| **52** | 43–2328 | 2,286 |
| **54** | 43–911 | 869 |
| **57** | 43–879 | 837 |
| **59** | 43–864 | 822 |
| **66** | 43–861 | 819 |
| **67** | 43–858 | 816 |
| **73** | 43–852 | 810 |
| **75** | 43–843 | 801 |
| **81** | 43–741 | 699 |
| **94** | 187–741 | 555 |

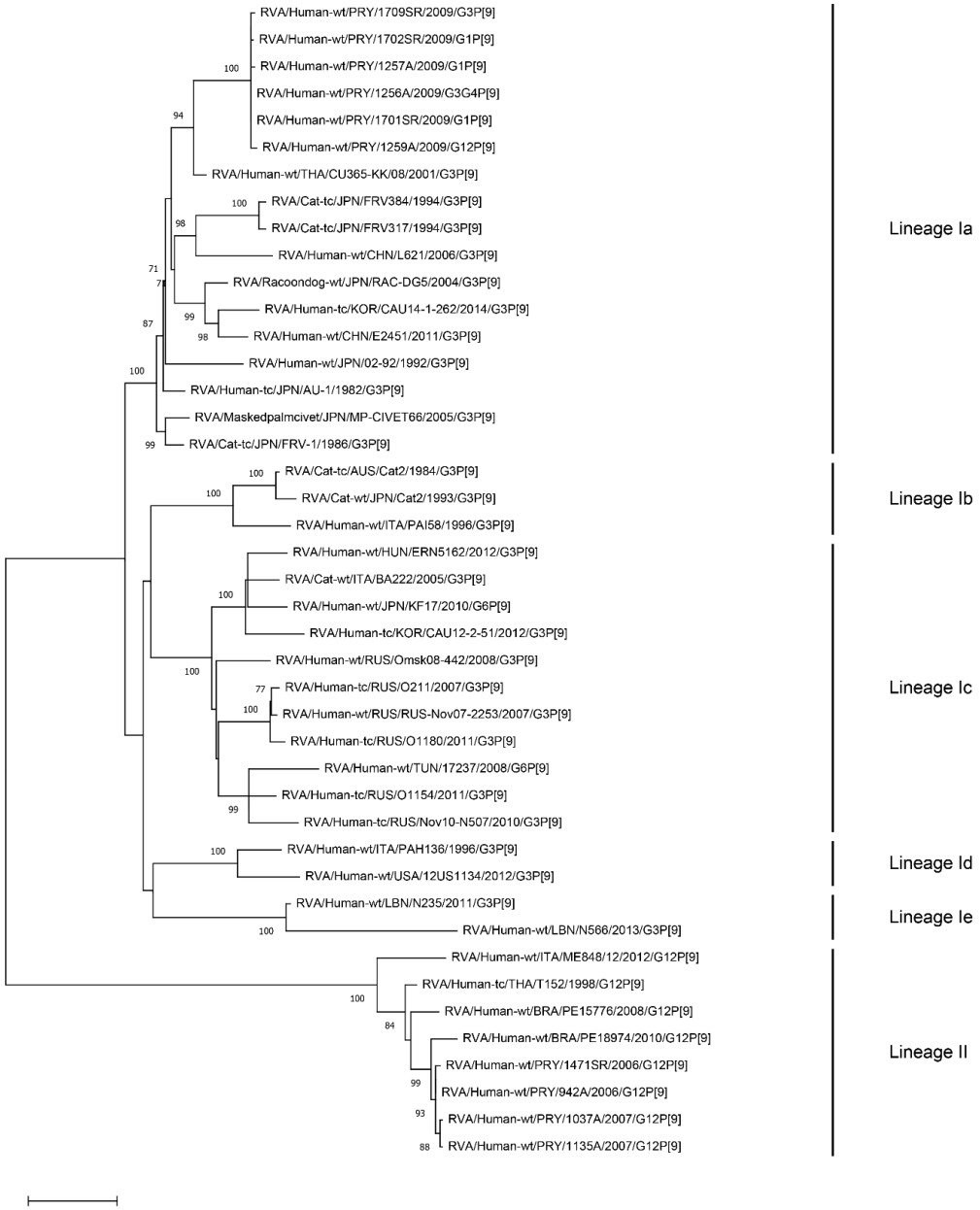


**Figure 1.** Phylogenetic tree constructed based on the full nucleotide sequences of the outer capsid protein VP4 genes of P[9] rotavirus strains. The numbers adjacent to the nodes represent the bootstrap values; values <70% are not shown. The scale bar shows genetic distance, which is expressed as nucleotide substitution per site.

**Table 3.** The distribution of different G and P[9] genotype combinations present in each rotavirus P[9] lineage when phylogenetic trees were constructed using full- (2,328) and partial-length (837) nucleotide sequences of outer capsid protein VP4 genes.

| Lineages | Full length nucleotide sequence (2,328 nt) | Partial length nucleotide sequence (837 nt) |
|---|---|---|
| **Ia** | G1P[9] | G1P[9] |
| | G3P[9] | G3P[9] |
| | G3G4P[9] | G3G4P[9] |
| | G12P[9] | G9P[9] |
| | | G12P[9] |
| **Ib** | G3P[9] | G3P[9] |
| **Ic** | G3P[9] | G3P[9] |
| | G6P[9] | G6P[9] |
| **Id** | G3P[9] | G3P[9] |
| **Ie** | G3P[9] | G3P[9] |
| **II** | G12P[9] | G12P[9] |

A total of 11 phylogenetic trees were constructed using different partial-length nucleotide sequences (Table 2) of P[9] strains. From partial-length nucleotide sequence of 837 nt and above consistently generated trees which could be classified into similar lineages and sub-lineages. The distribution of strains in each lineage and sub-lineage were consistent with the phylogenetic tree constructed using full- and partial-length gene sequences.

Overall, two lineages of P[9] strains could be identified in the phylogenetic tree constructed using 57 partial gene sequences (43–879 nt) of P[9] strains (Figure 2). Sub-lineage Ia consisted of G3P[9], G1P[9], G9P[9], G12P[9], and G3G4P[9] strain from Brazil, Japan, Paraguay, Thailand, China, , and Korea. The strains from Brazil, were of the G3P[9], G1P[9], and G9P[9] combination (Table 3).

G3P[9] combination was from Japan. Strains from Paraguay were G3P[9], G1P[9], G12P[9], and G3G4P[9]. All strains from Thailand, China, and Korea were G3P[9]. Sub-lineage Ib consisted of G3P[9] strains from Italy, Australia and Japan. Sub-lineage Ic consisted of genotype G3P[9], and G6P[9] from Russia, Hungary, Italy, Tunisia, Japan, and Korea. Russian strains were of the G3P[9], and G6P[9] combination. The strains from Hungary was of genotype G3P[9]. Italian strains were of genotype G3P[9] and G6P[9]. The strains from Tunisia and Japan were G6P[9], and from Korea were G3P[9]. Sub-lineage Id consisted of genotype G3P[9] from the USA, and Italy. Sub-lineage Ie consisted of strains from Lebanon of genotype G3P[9]. Lineage II consisted of strains from Paraguay, Brazil, Thailand and Italy. All strains were of the G12P[9] combination.

*Nucleotide identity*

Comparison of nucleotide identities of 43 full-length and 57 partial-length nucleotide sequences of the outer capsid protein VP4 gene among the two lineages and five sub-lineages of rotavirus P[9] are shown in table 4 and 5, respectively.

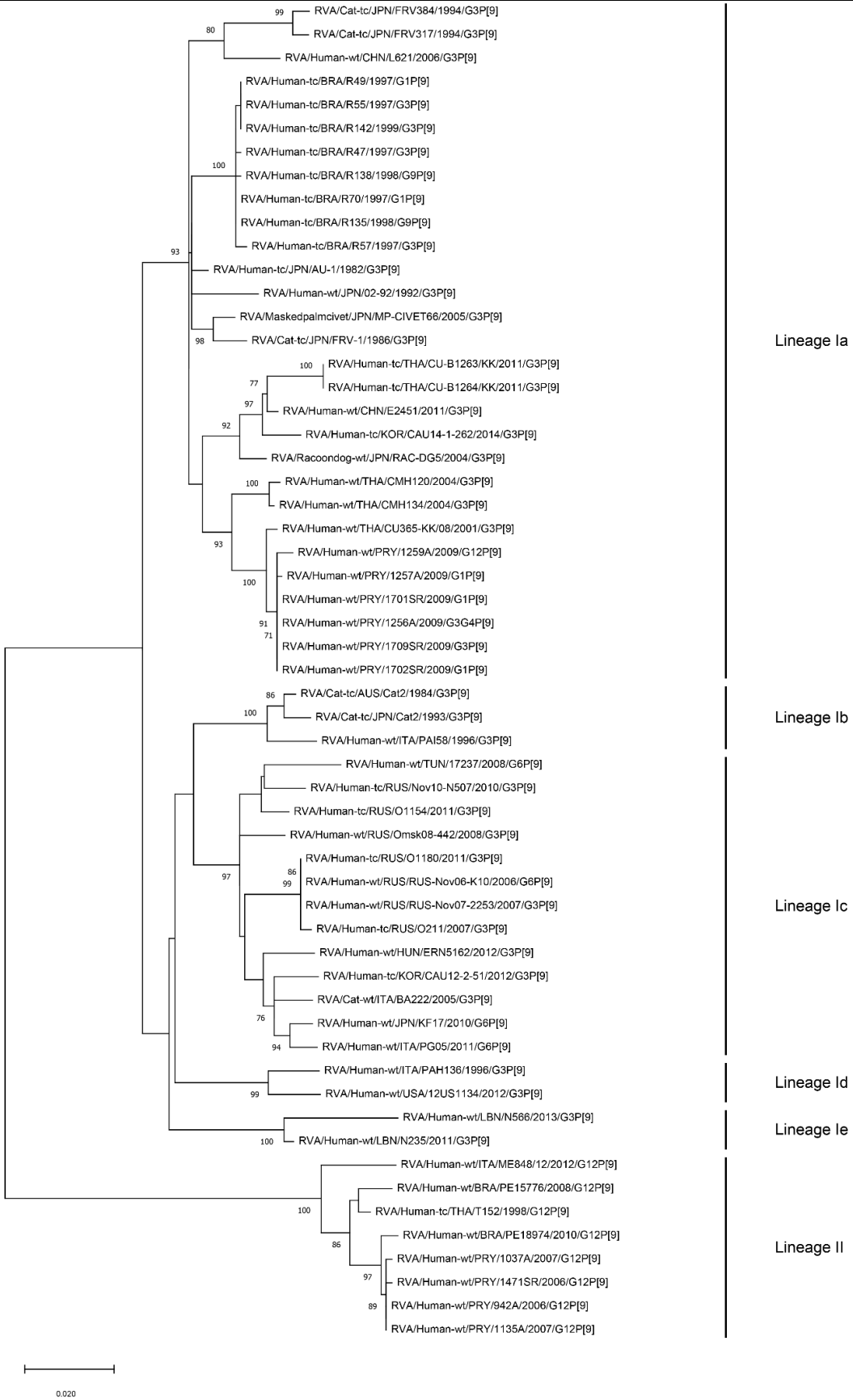


**Figure 2.** Phylogenetic tree constructed based on the partial nucleotide sequences of the outer capsid protein VP4 genes of P[9] rotavirus strains, with a length of 837 nucleotides. The numbers adjacent to the nodes represent the bootstrap values; values <70% are not shown. The scale bar shows genetic distance, which is expressed as nucleotide substitution per site.

**Table 4.** Comparison of nucleotide identities of 43 full-length nucleotide sequences of the outer capsid protein VP4 gene among the five lineages of rotavirus P[9].

| | Lineage Ia | Lineage Ib | Lineage Ic | Lineage Id | Lineage Ie | Lineage II |
|---|---|---|---|---|---|---|
| **Lineage Ia** | 96.2%–100% | 93.9%–95.7% | 94.0%–96.2% | 93.9%–95.7% | 91.9%–95.7% | 88.8%–90.5% |
| **Lineage Ib** | | 97.6%–99.5% | 94.0%–95.2% | 94.5%–95.0% | 92.1%–95.7% | 88.5%–89.4% |
| **Lineage Ic** | | | 96.2%–99.7% | 94.1%–95.1% | 91.5%–94.8% | 88.8%–89.9% |
| **Lineage Id** | | | | 97.8% | 92.1%–94.9% | 88.5%–89.0% |
| **Lineage Ie** | | | | | 96.6% | 87.6%–89.8% |
| **Lineage II** | | | | | | 97.2%–99.9% |

**Table 5.** Comparison of nucleotide identities of 57 partial nucleotide sequences of the outer capsid protein VP4 gene among the five lineages of rotavirus P[9].

| | Lineage Ia | Lineage Ib | Lineage Ic | Lineage Id | Lineage Ie | Lineage II |
|---|---|---|---|---|---|---|
| **Lineage Ia** | 95.5%–100% | 93.4%–95.8% | 93.3%–96.3% | 93.3%–95.2% | 92.5%–95.6% | 88.8%–90.8% |
| **Lineage Ib** | | 98.2%–99.2% | 94.9%–96.1% | 94.7%–95.1% | 93.1%–95.3% | 88.6%–89.7% |
| **Lineage Ic** | | | 96.2%–100% | 94.2%–95.6% | 92.7%–95.8% | 88.8%–90.6% |
| **Lineage Id** | | | | 97.9% | 93.1%–94.7% | 88.8%–89.4% |
| **Lineage Ie** | | | | | 97.5% | 88.2%–90.1% |
| **Lineage II** | | | | | | 97.0%–100% |

Full-length nucleotide sequences of P[9] strains of the sub-lineage Ia, Ib, Ic, Id, Ie, and II shared nucleotide identity of 96.2%–100%, 97.6%–99.5%, 96.2%–99.7%, 97.8%, 96.6%, and 97.2%–99.9% among themselves, respectively. The strains of different lineages showed a decrease in nucleotide identity (91.9%–99.7%), except for lineage II, which shared relatively low nucleotide identity (88.5%–90.5%) with strains of other lineages.

Partial-length nucleotide sequences of P[9] strains of the lineage Ia, Ib, Ic, Id, Ie, and II shared nucleotide identity of 95.5%–100%, 98.2%–99.2%, 96.2%–100%, 97.9%, 97.5%, and 97.0%–100% among themselves, respectively. The strains of different lineages showed a decrease in nucleotide identity (92.5%–96.3%), except for lineage II, which shared relatively low nucleotide identity (88.2%–90.8%) with strains of other lineages.

*Timeline of evolution*

The phylogenetic tree constructed using the Bayesian method also showed two lineages and five sub-lineages of P[9] strains (Figure 3), which corresponded with the lineages and sub-lineages as determined using the maximum likelihood method. All P[9] included in this study shared a common ancestor in circa 1864 (95% highest posterior density [HPD] circa 1755–1941) when lineage II diverged from lineage I. The strains in lineage II started evolving in circa 1983 (95% HPD 1964–1993). Sub-lineage Ie diverged from sub-lineages Ia, Ib, Ic, and Id in circa 1932 (95% HPD 1896–1960). Sub-lineage Ia diverged from sub-lineages Ib, Ic, and Id in circa 1945 (95% HPD 1919–1964) and different strains of sub-lineage Ia evolved in circa 1969 (95% HPD 1958–1977). Sub-lineage Id diverged from sub-lineage Ib, and Ic in circa 1948 (95% HPD 1923–1966 years). Sub-lineage Ib diverged from sub-lineage Ic in circa 1953 (95% HPD 1929–1970), and the strains in sub-lineage Ic started evolving in circa 1983 (95% HPD 1969–1994).

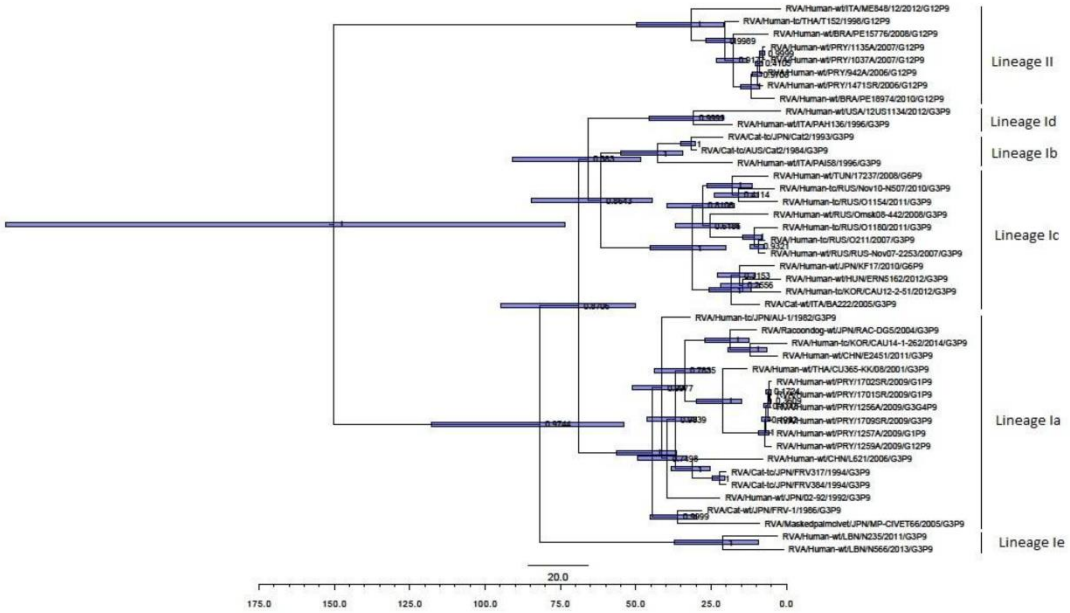


**Figure 3:** Bayesian maximum credibility tree showing the ancestry of rotavirus obtained by analyzing full sequences of the outer capsid protein VP4 gene of P[9] rotavirus strains. The nodes correspond to mean age at which the lineages/sub-lineages were separated from their most common recent ancestor; blue horizontal bars at the nodes represent the 95% highest posterior density of the most common recent ancestor. The numbers at the main nodes represent posterior values. The horizontal-axis at the bottom represents the time scale in years.

## DISCUSSION

All currently circulating P[9] strains were divided into two lineages and five sub-lineages, except for sub-lineage Ie, the strains in each lineage and sub-lineage were from multiple countries on different continents. This suggests that strains in a lineage do not belong to a specific geographical area. This might exemplify the role of human migration in the spread of strains in different countries. Humans tend to bring their accompanying animals, which might include domestic cats, during migration, and as human migration is a continuous process, this trend is expected to continue in future.

The results of this study confirmed sequences of the VP4 gene as short as 837 nt were adequate for lineage or sub-lineage designation. For new strains, it is still recommended where possible to use full-length nucleotide sequences for phylogenetic analysis to provide a more robust and comprehensive for analysis of the evolution, spread, and genome-wide heterogeneity of a given virus. In the present study, full-length nucleotide sequences were used during timeline evolutionary analysis because different portions of the gene have different rates of evolution, which might affect the determination of lineage age.

Sub-lineage Ia contained the AU-1 strain (G3P[9]), which was the first detected P[9] strain in humans (9). The Brazilian strains were from several outbreaks (21); these strains were also clustered in this sub-lineage and shared high nucleotide identity with AU-1 (28). All Paraguayan P[9] strains of sub-lineage Ia were detected in the same year; however, the P[9] genotype was in combination with different G genotypes. By contrast, the Paraguayan strains of lineage II were all G12P[9]. These were considered emerging strains (19), suggesting the possibility of outbreak. The results of the present study support the finding that Paraguayan strains share 99% nucleotide identity with T152, another G12P[9] rotavirus strain discovered in Thailand (10). However, the underlying mechanism for the specific combination of the P[9] from lineage II with G12 rather than G3 requires further study.

When full nucleotide sequences were compared, the P[9] strains of the same lineage and sub-lineage shared close nucleotide identity among themselves. When strains of different lineages and sub-lineages were compared, a decrease in nucleotide identity was observed, except for lineage II, which shared relatively low nucleotide identity with strains of other lineages. All of the strains in lineage II were G12P[9]; the significance of this genotype combination on nucleotide identity requires further study. Also, five of eight strains in lineage II were from South America and formed a cluster, which suggests the possibility of strains descended from a single ancestor are spreading across the continent.

Few differences were seen when the identities of partial-length sequences were compared with those of full-length sequences. Such differences in nucleotide identity using partial-length nucleotide sequences might be acceptable when no full-length sequences are available.

The information obtained in this study indicates that the origin of the common ancestor of currently circulating P[9] rotavirus strains might be too recent. We postulate that there could have been several rotavirus transmission events from cats to humans; however, older strains might have been wiped out by evolutionary constraints, and the currently circulating strains that evolved from a common ancestor in circa 1864 which could have survived and dispersed in different places with further local evolution. Because of increased human–animal interaction in recent decades it is possible that the current strains might evolve further and give rise to virulent strains of rotavirus.

Human–animal interaction has increased in recent years for several reasons, such as more humans having pets, a loss of animal habitats because of deforestation, and increase in large-scale farming and ecotourism. As a result, the potential for zoonotic transmission of viruses has increased, which could lay the foundation for the emergence of reassorted strains. The development of a common P[9] vaccine for humans and cats might help control rotavirus infection by this reassorted strain.

## CONCLUSION

We conclude that the VP4 gene of the available P[9] strains could be divided into two lineages. VP4 gene as short as 837 nt were adequate for lineage and sub-lineage designation. Although humans have had interactions with cats for thousands of years, the common ancestor of the current P[9] strain came into exist only 156 years ago. We also found that none of the lineages and sub-lineages has a specific region of distribution. One of the limitations of this study is that many of the genes in the analysis were sequenced from strains that have undergone passage in cell culture which might have had an impact on the analyses. Further study is needed particularly using nucleotide sequences of P[9] strains from cats to evaluate the time-line of evolution of P[9] strains.

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**Conflict of interest**
No conflict of interest was declared by the authors.

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