ORIGINAL ARTICLE

# Molecular characterization of a rare G9P[23] porcine rotavirus isolate from China

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**Abstract** The fifth most important G genotype, G9 rotavirus, is recognized as an emerging genotype that is spreading around the world. Sequence analysis was completed of a rare group A rotavirus, strain G9P[23], that was designated rotavirus A pig/China/NMTL/2008/G9P[23] and abbreviated as NMTL. It was isolated from a piglet with diarrhea in China. Nucleotide sequence analysis revealed that the VP7 gene clustered within the G9 lineage VId. The VP4 gene clustered within the rare P[23] genotype. NMTL is the first porcine G9 stain reported in China. Thus, to further characterize the evolutionary diversity of the NMTL strain, all gene segments were used to draw a phylogenetic tree. Based on the new classification system of rotaviruses, the NMTL sequence revealed a G9–P[23]–I5–R1–C1–M1–A8–N1–T1–E1–H1 genotype with close similarity to human Wa-like and porcine strains. The results showed that (i) NSP2 and NSP4 genes of NMTL exhibited higher genetic relatedness to human group A rotaviruses than to porcine strains, (ii) the VP2 and VP4 genes clustered with porcine and porcine-like human strains, and (iii) VP1 genes clustered apart from the Wa-like human and porcine clusters. In view of rotavirus evolution, this report provides additional evidence to support the notion that the human and porcine rotavirus genomes might be related.

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## Introduction

Group A rotaviruses (GAR), family *Reoviridae*, are a major cause of severe diarrhea in young humans and animals [5, 6]. More than 125 million infants and young children develop rotavirus diarrhea globally each year, resulting in 440,000 deaths among children less than 5 years of age, mostly in developing countries [15]. In China, it is estimated that there are 35,000 deaths per year, or one death per 500 children born, because of rotavirus [16]. Mature virus particles consist of an 11-segmented double-stranded RNA genome, which encodes six structural and six nonstructural proteins [5]. Based on the nucleotide sequences of the VP7 capsid protein gene, 24 G-genotypes have been identified. At least 34 P-genotypes, based on the VP4 capsid protein, have been reported from humans and animals [1, 21]. A notable characteristic of rotavirus strains is that gene segments are prone to genetic reorganization in nature or under experimental conditions. The most adaptable among the current reassortant human strains is the human G9 rotavirus. The G9 rotavirus is recognized as an emerging genotype that is spreading around the world [14, 18]. The rapidly increasing detection of this virus, in association with its genetic heterogeneity, raises questions regarding its origin and epidemiological importance. In mainland China, the first human G9 strain was detected in 1994 and was then isolated sporadically during the following years. From 2000 to 2007, G9 strains were not commonly detected [3].

In the present study, we obtained a porcine GAR strain, rotavirus A pig/China/NMTL/2008/G9P[23] (NMTL), isolated from a piglet with diarrhea in China. We determined the full-length nucleotide (nt) sequences of 11 gene segments of strain NMTL. The full genome of NMTL was analyzed at the nt level following the scheme proposed by the Rotavirus Classification Working Group (RCWG) [9, 10]. Sequence analysis showed that NMTL belongs to the G9 genotype combination with the rare P[23] genotype. To date, to our knowledge, this G–P combination was not seen frequently in pigs, and NMTL is the first porcine G9 isolated in China. In order to properly understand the origin of the G9P[23] genotype, we studied the genetic relatedness of the full genome of NMTL to those available as reference strains. Nucleotide sequence BLAST analysis of the VP1, NSP2 and NSP4 genes of NMTL revealed a higher degree of sequence identity to human strains than to porcine strains, prompting us to characterize all of the 11 gene segments of NMTL. Our findings provide important insights into the possible patterns of the evolution of G9 rotaviruses.

## Materials and methods

### Virus

The Group A rotavirus strain NMTL was isolated from stool specimens from a Chinese piglet with acute diarrhea and was propagated in MA104 cells in the presence of trypsin as described earlier [2, 13]. MA104 cells were grown in Eagle's MEM supplemented with 10 % calf serum, tryptose phosphate broth, and antibiotics. The isolated virus was purified three times from plaques and identified by electron microscopic observation.

### RT-PCR

For RT-PCR, virus RNA was extracted using a QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Germany), following the manufacturer's instructions. The forward and reverse primers used for the amplification of different gene segments were designed based on alignments of known 5' and 3' sequences of the respective gene segments found in the GenBank database. The PCR procedure was carried out with denaturation at 95 °C for 5 min, 30 cycles of amplification (10 s at 98 °C, 30 s at 55 °C, and 3 min at 72 °C), with a final extension of 10 min at 72 °C. The PCR amplicons were purified using a QIAquick PCR Purification Kit (QIAGEN/Westburg). The purified PCR amplicons were cloned into the PMD18-T vector (Takara) using molecular cloning techniques. Six clones were sequenced for each amplicon.

### Sequence analysis

The sequences were assembled, edited and analyzed using the SeqBuilder module of the DNASTAR software package (Lasergene, Madison, USA). Preliminary analysis was accomplished by comparison with the sequences available in the database using the web-based program BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic trees were constructed by the neighbor-joining method using MEGA software (version 4.1). Bootstrap analysis was performed based on 1000 replicates, and phylogenetic distances were measured by maximum composite likelihood. Genotypes were assigned to the 11 gene segments of strain NMTL following the RCWG classification scheme [9, 10]. For a given gene segment, only published strains with full-length, nearly full-length or complete open reading frame nt sequences were included in the analysis.

### Nucleotide sequence accession numbers

The nucleotide sequences of the 11 gene segments of the NMTL strain were submitted to the GenBank database and assigned the consecutive nucleotide sequence accession numbers JF781158- JF781168.

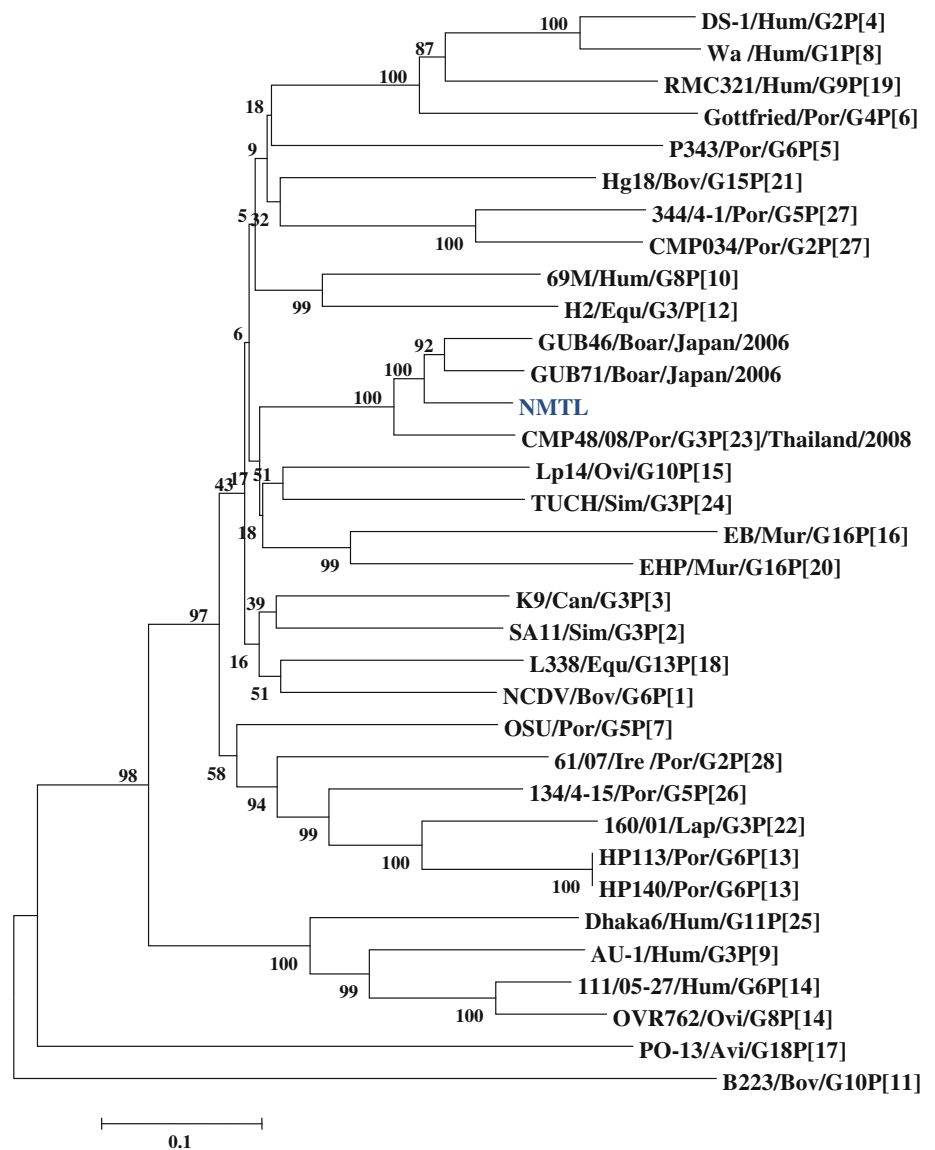## Results

### Nucleotide sequence analysis of strain NMTL

#### VP7

Currently, G9 rotaviruses are classified into six genetic lineages (I toVI) [17]. A phylogenetic tree based on the open reading frame of VP7 gene sequences showed that the China G9 clustered into the genetic lineage of VI, sharing 92.9–95.8 % identity at the nucleotide level with previously reported G9 strains detected in China and Japan, and USA A2 strains from the 1980s. The NMTL isolate and the Japanese porcine strain JP32-4 clustered into the genetic lineage VId. Five human G9 reference strains, K-1, 99-SP1904VP7, 99-SP1542VP7, 99-TK2082VP7, and 99-TK2091VP7, were their nearest phylogenetic neighbors, sharing 94.3-95.8 % identity at the nucleotide level. In contrast, NMTL showed homology ranging from 88.9 to 89.1 % with the G9 prototype strains Wi61, AU32 and F45, which were isolated from infants in the 1980s in America and Japan (Supplementary Fig. 1).

#### VP4

The VP4 gene of NMTL exhibited a maximum nt (88.4 %) identity with a cognate gene of strain GUB46 and shared

**Fig. 1** Phylogenetic analysis of the VP4 nt sequences of strain NMTL (JF781161), indicating its genetic relationship to strains representing the 27 P-genotypes. Abbreviations used for sequence analysis are as follows: hum, human; por, porcine; avi, avian; mur, murine; sim, simian; ovi, ovine; equ, equine; bov, bovine; fel, feline; can, canine; lap, lapine; lam, lamb; and out, outgroup



*VP1-3 and NSP1-5*

The VP1, NSP2, and NSP4 genes of strain NMTL shared higher nt identities with those of Wa-like human strains than with porcine strains (Supplementary Fig. 3, Figs. 3, 4), while the VP2, VP3, NSP1, NSP3 and NSP5 genes exhibited higher identities with those of porcine strains (Fig. 2, Supplementary Figs. 4–7). The VP1, VP2, NSP3 and NSP5 genes of NMTL exhibited higher nt identity of 91.2-97.5 % with the RU172 strain (G12 serotype), which is recognized as an emerging genotype [7, 8].

Phylogenetic analysis of the VP1-4, VP6-7 and NSP1-5 genes of NMTL revealed their overall genetic relatedness to Wa-like human and porcine strains. The following three observations were made about the respective genotypes: (i) the NSP2 and NSP4 genes of NMTL clustered with Wa

low identities of 32–76.1 % with strains representing the remaining 27 P-genotypes. This genetic relatedness was confirmed by phylogenetic analysis of VP4, where strains GUB46, GUB71 and CMP48/08 clustered within VP4 genotype P [23], away from the remaining 27 P-genotypes (Fig. 1).

*VP6*

Analysis of the VP6 gene of the NMTL strain revealed higher identity (94.9 %) with the human strain R479 than with porcine group A rotaviruses, with identities of 84.5–94.1 %. The NMTL strain clustered with the human G4P[6] R479 strain from China and appeared to be distantly related to the porcine cluster within the VP6 genotype I5 (Supplementary Fig. 2).

Fig. 2 Phylogenetic analysis of the VP2 nt sequences of strain NMTL (JF781159), indicating its genetic relationship to strains representing the five C-genotypes. Abbreviations used for sequence analysis are as follows: hum, human; por, porcine; avi, avian; and sim, simian
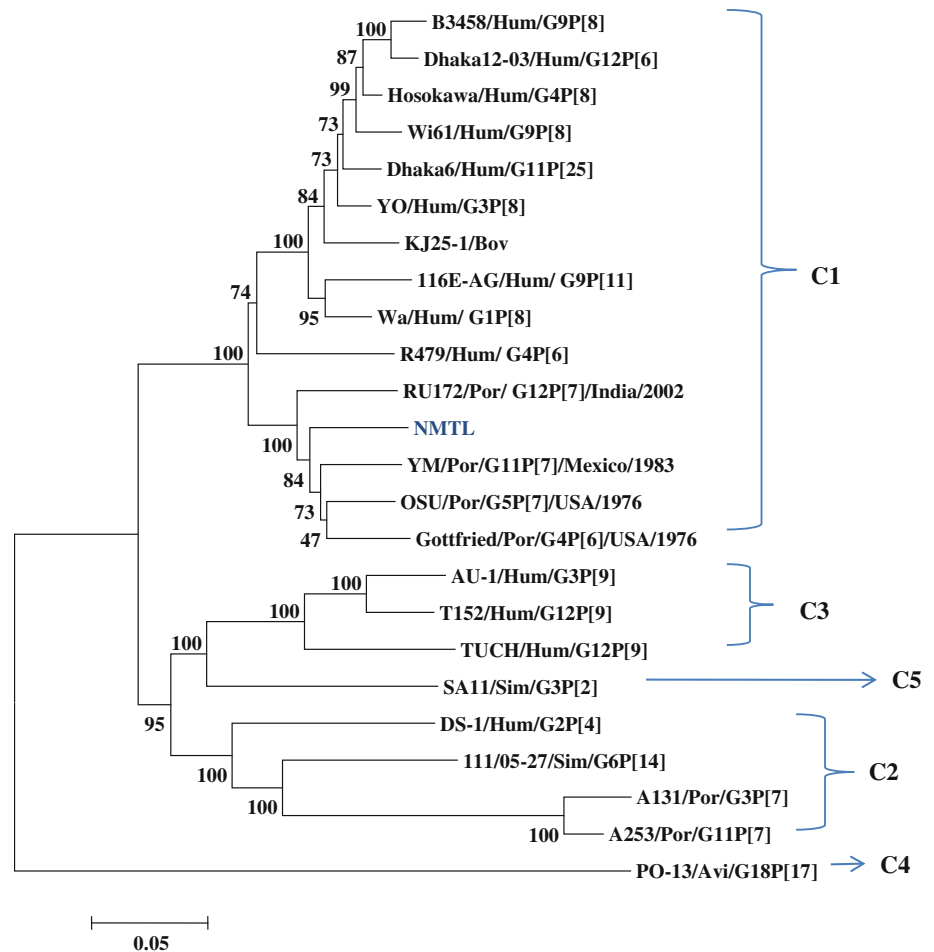
and Wa-like human GARs, including G9 strains within the cluster, but away from the porcine cluster; (ii) the VP2 and VP4 genes clustered with porcine and porcine-like human strains; and (iii) the VP1 genes clustered apart from the Wa-like human and porcine clusters. The phylogenetic relationships between the NMTL strain and the other viruses in the trees are consistent with the hypothesis that the NSP2, NSP4 and VP1 genes in the NMTL virus are of human origin; however, the species origin cannot be inferred from the phylogenetic trees estimated with the other gene sequences because the human and porcine strains were more closely related phylogenetically to NMTL. Considering (1) the probable human origin of the NSP2 and NSP4 genes of the NMTL strain, (2) the porcine nature of the VP2 and VP4 genes of strain NMTL, and (3) the close genetic relatedness between the NMTL strain and the Wa-like human strains in other genes, it might be tempting to suggest that (i) the porcine NMTL strain might have originated from porcine–human reassortment events, or alternatively, (ii) the Wa-like human and porcine G9 strains might have originated from a common ancestor and eventually evolved (by genetic drift and shift) with time.

Full-genome-based classification of strain NMTL

Complete genotype analysis of strain NMTL was done according to the percentage identity at the nucleotide level of 11 gene segments as per the newly proposed nomenclature for rotaviruses [9, 10]. Based on nucleotide identities of greater than 80 % between NMTL and established genotype A rotavirus strains, strain NMTL clusters with the G9–P[23]–I5–R1–C1–M1–A8–N1–T1–E1–H1 genotype, representing the gene segments VP7–VP4–VP6–VP1–VP2–VP3–NSP1–NSP2–NSP3–NSP4–NSP5/6.

## Discussion

Group A rotaviruses are one of the major causes of severe gastroenteritis in young children and other animals [12, 20]. Vaccination is the only control measure likely to have a significant impact on the incidence of severe dehydrating rotavirus disease [4]. This high disease burden has motivated major efforts to develop rotavirus vaccines. However, the high degree of genetic and antigenic variation among rotaviruses hinders the vaccine development programs [19].

**Fig. 3** Phylogenetic analysis of the NSP2 nt sequences of strain NMTL (JF781165), indicating its genetic relationship to strains representing the five N-genotypes. Abbreviations used for sequence analysis are as follows: hum, human; por, porcine; avi, avian; sim, simian; ovi, ovine; and bov, bovine
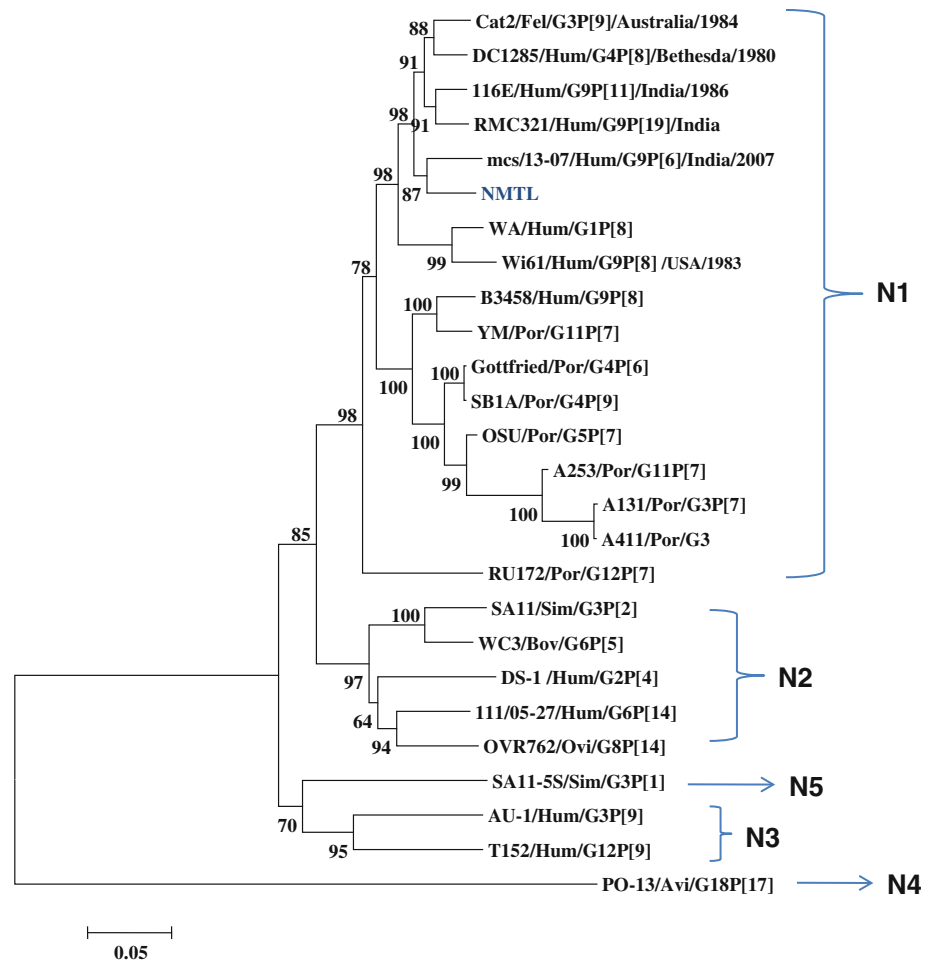
G9 rotavirus is recognized as an emerging genotype spreading around the world. These serotype strains have aroused considerable scientific interest in various aspects of the G serotype, including the epidemiology, evolutionary origin, and genetic composition of the virus, as well as development of vaccines against this serotype. By phylogenetic analysis, G9 strains have been classified into six distinct lineages (I–VI): lineage I, strains detected in the 1980s in the United States and Japan; lineage II, strains detected in 1986 and found exclusively in India; lineage III, strains that emerged/reemerged in the mid-1990s; lineage IV, strains detected in 1997 exclusively in China; lineage V, strains detected in 1997–1998 exclusively in the United States; and lineage VI, strains detected in 1997–2002 [17]. Lineages I, II, IV and V were found only in humans, but lineages III and VI were found in both humans and pigs. A surprising observation was that a number of the Japanese and Chinese porcine and human G9 strains were placed in a monophyletic cluster within lineage VI and were further classified into seven sublineages. Of these, sublineages a–d and g were found only in pigs, and sublineages e and f were detected in humans [17].

In mainland China, the first human G9 strain was detected in 1994 and then isolated sporadically during the following years, but during 2000–2007, G9 strains were uncommon [3]. G9 strains previously identified in Lulong, Suzhou, Chongqing and Kunming from 1998 to 2005 accounted for 0.6 %, 1.2 %, 4 % and 5 % of rotavirus isolates, respectively [22].

From 2006 to 2007, G9 accounted for 1.4 % (6/494) of rotavirus isolates in Lanzhou and 4.4 % (12/272) in Lulong. The low detection frequency of G9 in mainland China is puzzling, given its greater prevalence worldwide [3]. The isolate NMTL and the Japanese porcine strain JP32-4 clustered into genetic lineage VId. The NMTL strain shared the maximum nucleotide identities of 94.3-95.8 % with five human G9 strains, K-1, 99-SP1904VP7, 99-SP1542VP7, 99-TK2082VP7 and 99-TK2091VP7. Moreover, sequence analysis of the VP4 gene showed that NMTL clusters with the rare P[23] genotype. On the other hand, phylogenetic analysis showed that the NSP2 and NSP4 genes of strain NMTL appeared to be of human origin and the VP2 and VP4 genes to be of porcine origin. In view of NMTL rotavirus evolution, this report provides

**Fig. 4** Phylogenetic analysis of the NSP4 nt sequences of strain NMTL (JF781167), indicating its genetic relationship to strains representing the 11 E-genotypes. Abbreviations used for sequence analysis are as follows: hum, human; por, porcine; avi, avian; and mur, murine
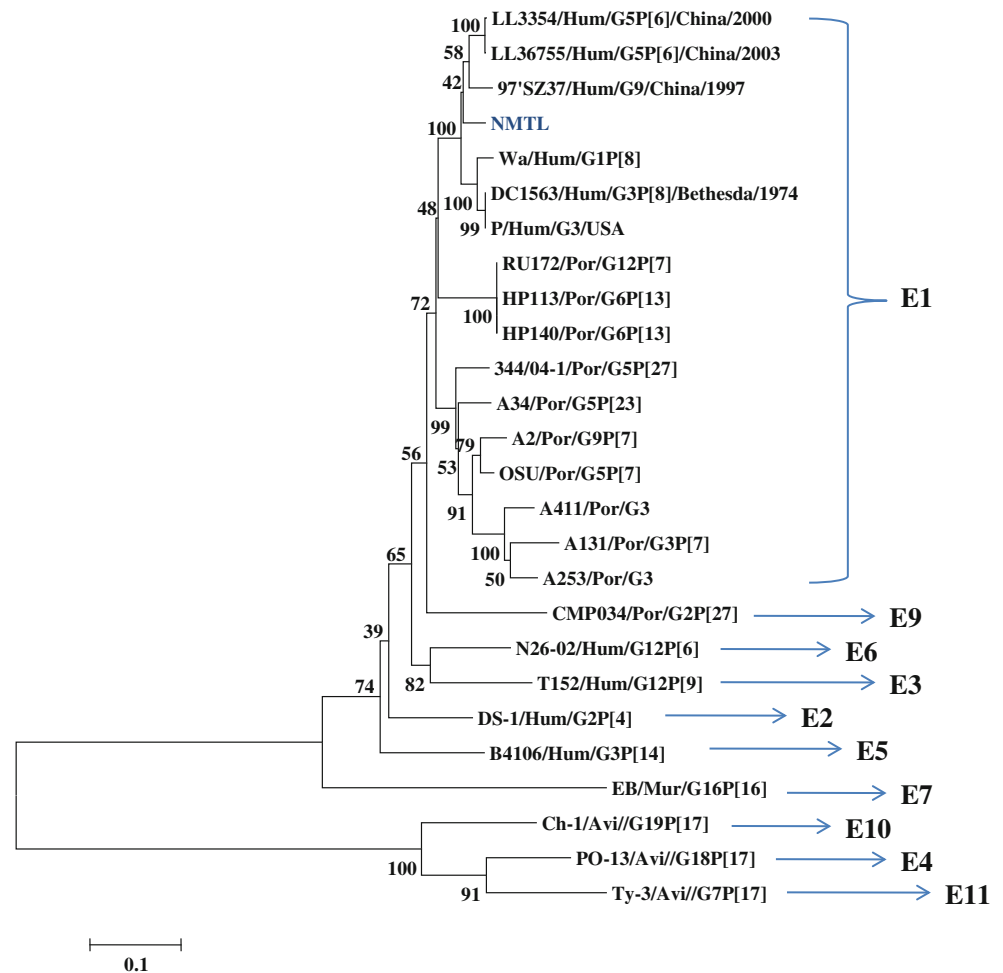


additional evidence to support the hypothesis that a genomic relatedness exists between human and porcine G9 rotaviruses. Molecular characterization of strain NMTL showed yet another example of the constant flux of genetic material between the human and gene pools of other animal rotaviruses, frequently in developing countries where humans and animals live in close proximity. For the G9 rotavirus, the origin is unclear, although pigs are suspected to be a potential host reservoir for these genotypes, as this is the only species, besides humans, from which G9 RVs have been isolated to date [11]. The current data suggest the occurrence of interspecies transmissions of RVs between human and porcine hosts, but the detection of G9 porcine rotaviruses has been reported only in the United States, Brazil and Japan. NMTL is the first porcine G9 strain that was reported in China. The dearth of information on porcine G9 rotavirus prevents us from being able to pinpoint the original host of origin for the G9 rotavirus strains, and increased surveillance in animals, especially in pigs, will be necessary to answer this question.

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