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Cloning and Nucleotide Sequence Analyses of 11 Genome Segments of Two American and One British Equine Rotavirus Strains

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Abstract

Group A equine rotavirus (ERV) is the main cause of diarrhea in foals and causes severe economic loss due to morbidity and mortality on stud farming worldwide. Molecular evolution of equine rotaviruses remains understudied. In this study, whole-genomic analysis of 2 group A ERV, FI-14 (G3P[12]), H-2 (G3P[12]) isolated from American, and FI23 (G14P[12]) from British was carried out and genotype constellations were determined as G3-P[12]-I6-R2-C2-M3-A10-N2-T3-E2-H7 for FI-14; G14-P[12]-I2-R2-C2-M3-A10-N2-T3-E2-H7 for FI23; and G3-P[12]-I6-R2-C2-M3-A10-N2-T3-E2-H7 for H-2, respectively. With the exception of the VP7 and VP6 gene, 2 G3P[12] strains (FI-14 and H-2) and one G14P[12] strain (FI23) were highly related genetically. Of note, the VP6 genotype of H-2 strain was previously reported to be I2, however, sequence and phylogenetic analyses demonstrated that it was I6. Therefore, it showed that G3P[12] ERV strains and G14P[12] ERV strains bore a distinct VP6 genotype: I6 for G3P[12] strains and I2 for G14P[12] strains. Moreover, it demonstrated that T-cell epitope 299P-300P/Q residues (PP/Q) of VP6 may be considered as I2 ERV typical molecular marker, which facilitates the analysis of the molecular evolution of equine rotaviruses.

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Conflicts of interest

We declare that we have no conflicts of interest.

Introduction

Rotaviruses form a genus in the *Reoviridae* family and are classified into 7 distinct groups (A to G) or a tentative H group according to VP6 gene sequences. Group A rotaviruses (RVA) are major pathogens associated with acute gastroenteritis in humans and animals (Kapikian, 2001).

Rotavirus genome consists of 11 segmented double-strand RNA (dsRNA), being enclosed in a triple-layered capsid. The VP7 and VP4 genes, encoding outer capsid, are used to classify rotaviruses into G and P genotypes, respectively (Kapikian, 2001). Group A equine rotavirus (ERV) is the major cause of diarrhea in foals up to 3 months of age and the acute dehydration can incur severe economic burden due to morbidity and mortality in studs (Bailey et al., 2013; Ghosh et al., 2013; Papp et al., 2013). The G3P[12] and G14P[12] are the most prevalent equine rotavirus strains worldwide (Ghosh et al., 2013; Papp et al., 2013).

ERV FI-14 strain (RVA/Horse-tc/USA/FI-14/1981/G3P[12]) was identified to be G3P[12] genotype and was characterized to bear both subgroup I and subgroup II specificities. However, the whole genome sequence of ERV FI-14 strain was unavailable except segments 4, 5, 6 and 10 (Hoshino et al., 1987). Strain FI23 (RVA/Horse-tc/USA/FI23/1981/G14P[12]) was originally isolated from a diarrheic foal and only segments 4, 5, 6, 9 and 10 were sequenced (Taniguchi et al., 1994). H-2 strain (RVA/Horse-tc/GBR/H-2/1976/G3P[12]) was isolated from a Prezewalski foal and identified as a G3P[12] strain and sequences of segments 4, 5, 6 and 10 were determined (Ciarlet et al., 2000). Until now, the unavailability of whole-genome sequences of equine rotaviruses of epidemiologic importance impedes us to analyze the characteristics, evolution or possible interspecies transmission.

In 2009, a novel nomenclature system was proposed by a Rotavirus Classification Working Group (RCWG) based on complete nucleotide sequences of all 11 genomic RNA segments (Matthijnssens et al., 2008a; Matthijnssens et al., 2011; Matthijnssens et al., 2008b). Here we report the complete genome classification of 2 American strains, FI-14 and FI23 and one British strain H-2ERV based on all the 11 segment sequence data by using RotaC v2.0 (Maes et al., 2009).

Materials and methods

RNA samples of ERV FI-14, FI23 and H-2 were provided by Yasutaka Hoshino (NIH) and polyadenylated using A-plus Poly(A) Tailing Kits (Epicentre Biotechnologies, USA) as described previously (Meis, 2006) and the cDNAs were synthesized using oligodT primer with the SuperScript II RT kit (Invitrogen) as described previously (Cook and McCrae, 2004). PCR specific primers were designed according to GenBank database or published data (Tsugawa and Hoshino, 2008).

The sequencing of rotavirus segments was performed with the BigDye terminator v3.1 cycle sequencing reaction kit (Applied Biosystems Group) on an ABI 3730 DNA analyzer (Applied Biosystems Group). Sequence data were analyzed using Sequencher 4.7 (Gene Codes Corporation) or BLAST (NCBI) program. Phylogenetic analysis was performed using MEGA6 software (Tamura et al., 2011; Tamura et al., 2013). The nomenclature of the 3

ERV strains were determined using the RotaC 2.0 (<http://rotac.regatools.be/>) (Maes et al., 2009). The RT-PCR and sequencing primers are listed in supplementary Table S1.

GenBank accession numbers of three ERVs

The VP1 to NSP5 of ERV strain FI14 are KM454481- KM454491; H-2 VP1 KM454492; H-2 VP2 KM454493; H-2 VP3 KM454494; H-2 VP4 KM454495; The VP1 to NSP5 of ERV strain H-2 VP1-NSP5 are KM454492- KM454502; The VP1 to NSP5 of ERV strain FI23 are KM454503-KM454513.

Results

According to all the 11 genomic RNA segment sequences, a novel nomenclature for the 3 ERV strains were genotyped with RotaC v2.0b tool (<http://rotac.regatools.be/>) (Maes et al., 2009) and the complete genotype constellations were showed in Table 1. The genome configuration of FI23 strain was distinct from the other 2 G3P[12] strains, with G14 VP7 genotype and I2 VP6 genotype (Table 1). Similar to all other reported ERV strains, the 3 ERV strains shared the P[12], C2, M3, A10, N2, T3 and H7 genotypes. The I6 genotype was associated with G3 genotype and the I2 genotype was associated with G14 genotype (Table 1). This finding was conflicted with a previous study where I6 genotype of the G3P[12] strain H-2 was reported as I2 (Matthijnssens et al., 2012).

The VP7 sequences of FI-14 and H-2 formed a phylogenetic genotype cluster for sharing higher than 98% identity (99%) and formed a sub-genotype with other 7 Japanese ERV strains. Obviously, the G3 genotype divided into Japanese sub-lineage (G3a) and Argentine sub-lineage (G3b). FI23 was separated to a single phylogenetic genotype cluster because it shared less than 84% identity (82.4%-83.4%) with FI-14 and H-2 strains (Fig. 1A). The G14 genotype strains were divided into 2 sub-lineages, Argentine sub-lineage (G3a) and Japanese sub-lineage (G14b). The results suggested that G3P[12] and G14P[12] ERV strains were the dominant epidemic strains in equine.

The VP4 nucleotides of FI-14, FI23 and H-2 shared higher than 98.8% identity (98.8%-99.7%) and formed a phylogenetic genotype cluster with most of Japanese ERV strains and the 3 Argentine ERV strains also formed a single P[12] sub-genotyped. The other 4 ERV strains, including 2 German strains, shared closer homology with the 3 Argentine ERV strains than that of Japanese strains. The results indicated that FI-14, FI23 and H-2 bearing closer genetic similarity with Japanese phylogenetic genotype branch on the basis of G VP7 genotype and P VP4 genotype (Fig. 1B).

The VP6 sequence of FI-14 and H-2 was different from other VP6s and FI-14 and H-2 formed a phylogenetic genotype cluster for sharing higher than 90% identity (90.7%-99.4%) as I6 sub-genotype. However, the strain H-2 was reported as I2 previously (Ghosh et al., 2012; Ghosh et al., 2013; Matthijnssens et al., 2012). FI23 formed a single phylogenetic genotype cluster as I2 sub-genotype because it shared less than 80% identity (78.9%-79.3%) with FI-14 and H-2 (Fig. 1C). Obviously, most of I2 and I6 genotype ERV strains bore P[12] VP4 genotype.

The cognate genes encoding VP1 proteins of the 3 ERV strains shared a high nucleotide sequence identity (FI-14 and H-2 had 100% identity; FI-14, FI-23 and H-2 shared 94.8% identity) and were genotyped as R2 and formed a single sub-genotype. Therefore, FI-14, FI23 and H-2 formed a distinct phylogenetic sub-genotype cluster with Japanese strains (RVA/Horse-tc/JPN/HH-22/1989/G3P[12], RVA/Horse-tc/JPN/OH-4/1982/G6P[5]), and distinguished from Argentine and Irish ERV R2 genotype strains (Fig. 1D).

The sequence of VP2 of the 3 ERV strains shared a high sequence identity (80.8%-99.1%). FI-14, FI23 and H-2 strains formed a distinct phylogenetic genotype cluster and genotyped as C2. However, they shared closer relationship with Irish strain (RVA/Horse-wt/IRL/04V2024/2004/G14P[12]) and Japanese HH-22 (RVA/Horse-tc/JPN/HH-22/1989/G3P[12]) than that of Argentine ERV strains (Fig. 1E).

The sequence of VP3 of the 3 ERV strains shared a high sequence identity (78.1%-99.2%). FI-14 and FI23 formed a distinct phylogenetic genotype cluster and belonged to genotype M3. However, strain H-2 formed another sub-genotype M3 with Argentine and Irish ERV strains. The most of ERV strains bore P[12] VP4 genotype formed a sub-genotype (M3a) (Fig. 1F).

NSP1 sequences of FI-14, FI23 and H-2 ERV strains shared higher than 95% identity (95.2%-96.6%) and were genotyped as A10. However, they formed 2 different sub-genotypes phylogenetically. FI-14 assigned to a single phylogenetic sub-genotype with an Irish ERV strain (RVA/Horse-wt/IRL/03V04954/2003/G3P[12]) whereas FI23 and H-2 formed another sub-genotype with other A10 genotype ERV strains, including 2 Japanese strains (Fig. 2A).

NSP2 sequences of FI-14, FI23 and H-2 strains shared higher than 99% identity (99.3%-99.8%) and were genotyped as N2. FI23 formed a single branch. H-2 clustered in a sub-genotype with other Japanese HH-22 strain, and FI-14 clustered in another sub-genotype branch with Irish 03V04954 strain (Fig. 2B).

NSP3 sequences of FI-14, FI23 and H-2 strains shared higher than 98% identity (98.6%-100%) and assigned as T3. They formed a phylogenetic sub-genotype cluster with 2 Irish ERV strains and one of South Africa strain (RVA/Horse-wt/ZAF/EqRV-SA1/2006/G14P[12]) (Fig. 2C). Obviously, 3 Argentine ERV strains separated relatively far from FI-14, FI23 and H-2 (Fig. 2C).

NSP4 sequences of FI-14, FI23 and H-2 strains shared higher than 98% identity (98.8%-100%) and formed a single phylogenetic sub-genotype cluster and assigned as E2, which formed a cluster with 5 Japanese ERV strains as follows: RVA/Horse-tc/JPN/CH-3/1987/G14P[12], RVA/Horse-tc/JPN/BI/1981/G3P[12], RVA/Horse-tc/JPN/HH-22/1989/G3P[12], RVA/Horse-tc/JPN/OH-4/1982/G6P[5], RVA/Horse-wt/JPN/HI-23/1981/G3P[12] and separated from 3 Irish ERV strains, RVA/Horse-wt/IRL/04V2024/2004/G14P[12], RVA/Horse-wt/CM1/IRL/2008/G14P[X], RVA/Horse-wt/IRL/03V04954/2003/G3P[12] and one of South Africa strain (RVA/Horse-wt/ZAF/EqRV-SA1/2006/G14P[12]). However, 7 Argentine ERV strains formed distinct sub-genotype branch of E12. Of note, one of Argentine ERV strains (RVA/Horse-wt/ARG/E3198/2008/

G3P[3]) located in E3 genotype branch (Fig. 2D) and related to simian RRV (RVA/Simian-tc/USA/RRV/1975/G3P[3]) and feline/canine-like rotaviruses (Mino et al., 2013).

NSP5 sequences of FI-14, FI23 and H-2 strains shared higher than 99% identity (9.1%-100%) and formed a phylogenetic genotype cluster H7 with other ERV strains (Fig. 2E). However, they were distinctly separated from Argentine ERV strains and mostly closer to Japanese strain HH-22.

Taken together, the phylogenetic genotypes of FI-14, FI23 and H-2 strains showed a tendency that the G and P genotypes shared closer homology with most of Japanese ERV strains (especially strain HH-22) referenced in this study than that of Argentine or Irish ERV strains. On the other hand, the A and T genotypes of FI-14, FI23 and H-2 strains shared closer homology with most of Irish ERV strains. However, FI-14, FI23 and H-2 strains separated from Argentine ERV strains except for N2 genotype (NSP2).

The amino acid residues differentiation of ERV strains were clearly separated into G3 and G14 lineage. The alignment of the VP7 antigenic regions A (aa 87-101), B (aa 141-152), C (aa 208-224), and F (aa 235-242) (Nemoto et al., 2011) showed the distinct amino acid differentiation between G3 and G14 genotype strains (Supplementary Fig. S1). Obviously, the G3 type ERVs were also separated as Japanese group and Argentine group in reference to the antigenic regions A, C, and F amino acid residues.

Japanese G14 ERV circulating in the late 1990s are closely related to the FI23 strain which was isolated 1981 in North America. Therefore, the Japanese G14 ERV circulating in the late 1990s is likely to originate from the American G14 ERV in the 1980s (Nakagomi et al., 2003).

VP6 amino acid sequences alignment also showed genetically-distinct clades between I2 and I6 genotype ERV strains. Of note, residues 299 and 300 are deleted (or missing) in I6 genotype strains, but in I2 genotype strains there are 2 additional residues of 299P and 300P or 300Q (Supplementary Fig. S2). In order to elucidate the universality of residues 299P-300P/Q in I2 genotype of rotavirus strains, we further compared the VP6 sequences of I2 ERVs with other non-ERV I2 genotype strains. The results indicated that the residues of 299P and 300P or 300Q only existed in I2 genotype ERVs (Supplementary Fig. S3). Therefore, we considered residues 299P-300P/Q as I2 ERV typical amino acid marker: PP/Q residues.

The T-cell epitopes (amino acids 289-302) of VP6 was determined with functional mapping of murine rotavirus EDIM strain VP6 protein (Choi et al., 2000). The PP/Q residues are just inserted into T-cell epitopes region between residues 298P and 299N. After alignment analysis of the VP6 T-cell epitopes (289RLSFQLMRPPNMTP302) with other ERVs, we found that 86% amino acid residues of T-cell epitopes were conserved in all ERVs, except for 295I of ERV E404 and 300I of Lamb-NT (Supplementary Fig. S4).

VP7-VP6 interaction domain is important for virion assembling. The residues (163S to 167N, 313P, 310N) of VP6 and residues (58P to 62S, 279P, 281T, 305Q) of VP7 were indicated to be crucial for VP6-VP7 interaction (Chen et al., 2009). We analyzed the VP6

conserved residues of 3 ERVs in this study and other non-ERVs. The results suggested that these residues were conserved in all ERV strains except that 310Q in FI-14 ERV and the 313P in I2 genotype was replaced of 313Q in other I genotype VP6 (Supplementary Fig. S5). The residues of VP7 were also conserved except for 281I in G14 genotype ERVs (Supplementary Fig. S1).

Discussion

Rotavirus classification was historically based on the VP6, VP4 and VP7 serotypes, which was performed by cross-neutralization assays using immune sera (Kapikian, 2001). However, the lack of proper immune sera impeded classification of each novel rotavirus strain. As the development of the gene sequencing technology, a complement rotavirus classification method was developed, which was based on identities between sequences of cognate rotavirus gene segments of VP4, VP6, VP7, and NSP4 (Martella et al., 2006; Martella et al., 2003; Steyer et al., 2007). To date, more than 60 ERV strains have been determined for the complete open reading frames (ORF) sequences of all 11 genome segments and a novel rotavirus nomenclature system was developed based on the nucleotide cutoff value of each segment of 11 full genomic sequence data (Bailey et al., 2013; Ghosh et al., 2013; Matthijssens et al., 2008b; Matthijssens et al., 2012; Mino et al., 2013). However, aside from the genes encoding outer capsid proteins (VP7 and VP4) and the intermediate layer protein (VP6), little is known about the whole genomic information of ERV strains because there are few ERV's full-genome sequences have been determined (Matthijssens et al., 2012). Molecular evolution of equine rotaviruses remains understudied. The limited whole genome sequence data of ERV affects the understanding of the genetic relationship among ERV strains or with rotaviruses from other species.

The previous study have reported full genome analysis of 7 ERV strains including the FI23, FI-14 and H-2 ERV strains (Ghosh et al., 2013; Matthijssens et al., 2012). However, the sequence and genotype information of the 3 ERV strains were incomplete and the I6 genotype of the H-2 strain was reported as I2 (Ghosh et al., 2013; Matthijssens et al., 2012). This discrepancy needs to be clarified. The possibility cannot be excluded that there are multiple clones of H-2 isolates among laboratories. Alternatively, it also could not exclude the possibility of laboratory contamination during the cloning or the sequencing procedure. However, in this study, we identified that the I genotype of H-2 strain is I6.

There seems to be a possible linkage between VP6 and VP7. It was merited to further investigate whether the observed VP7-VP6 linkage was specific for ERV G3/G14P[12] strains. To study the possible involvement of gene(s) other than VP7 and VP6 gene in the observed VP7-VP6 genetic linkage, we analyzed the phylogeny of the remaining 9 genes of 2 G3P[12] strains and one G14P[12] strain sequenced in the present study as well as other G3/G14P[12] strains available in GenBank. The result showed that G genotype and P genotype of the FI-14, FI23 and H-2 strains shared high homology with most of Japanese ERV strains and the A genotype and T genotype shared close homology with most of Irish ERV strains. That suggested G3 ERV and G14 ERV strains each constituted a separate lineage (at least for VP6 lineage). A related finding was reported previously that the VP7 gene of the G3 ERVs circulating in Japan from 2003 to 2008 were classified as G3b sub-

lineage together with the Japanese vaccine strain HO-5 (isolated in 1982), JE75 and JE77, indicating that the VP7 gene of the G3b ERV in Japan has been highly conserved for a long time (Nemoto et al., 2011).

As for biological properties, the T-cell epitope of VP6 (289-302 aa) was functionally associated with RVA protective immune response. A murine VP6 fusion protein vaccine containing the T-cell epitope reduced virus shedding by 96% without any measurable rotavirus-specific antibody in mice (Choi et al., 2000). Therefore, we suggest that the additional 299P-300P/Q residues (PP/Q) of I2 genotype VP6 in the T-cell epitope may be considered as I2 ERV typical molecular marker, which facilitates the analysis of the molecular evolution of ERVs as well as specific immunological mechanisms. However, the PP/Q residues were not confirmed in other I2 genotype RVA strains (e.g. RVA/Human-tc/USA/DS-1/1976/G2P[4], RVA/Cow-tc/FRA/RF/ 1982/G 6P[1]). It will be interesting to investigate when and how ERV I2 genotype viruses obtained these additional residues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. The full genome sequences of FI-14, FI23 and H-2 were sequenced and genotyped.
2. Identified 299P-300P/Q residues (PP/Q) of VP6 as I2 ERV typical molecular marker.
3. PP/Q residues were found in the conserved T-cell epitope of ERV VP6.

Fig 1. Molecular phylogenetic analysis of structural viral nucleotides

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 700 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (700 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution

was used to model evolutionary rate differences among sites. The rate variation model allowed for some sites to be evolutionarily invariable. Codon positions included were 1st +2nd+3rd+Noncoding. Evolutionary analyses were conducted in MEGA6.

Fig 2. Molecular phylogenetic analysis of nonstructural viral nucleotides

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 700 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (700 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The rate variation model allowed for some sites to be evolutionarily invariable. Codon positions included were 1st +2nd+3rd+Noncoding. Evolutionary analyses were conducted in MEGA6.

Table 1

The genotyping of the three equine rotavirus strains in this study and compared with selected sequenced ERV group A reference strains

ERV Strains	Genotypes
* RVA/Horse-wt/ARG/E30/1993/G3P[12]	G3-P[12]-I6-R2-C2-M3-A10-N2-T3-E12-H7
* RVA/Horse-wt/IRL/03V04954/2003/G3P[12]	G3-P[12]-I6-R2-C2-M3-A10-N2-T3-E2-H7
RVA/Horse-tc/GBR/H-2/1976/G3P[12]	G3-P[12]-I6-R2-C2-M3-A10-N2-T3-E2-H7
RVA/Horse-tc/USA/FI-14/1981/G3P[12]	G3-P[12]-I6-R2-C2-M3-A10-N2-T3-E2-H7
RVA/Horse-tc/USA/FI23/1981/G14P[12]	G14-P[12]-I2-R2-C2-M3-A10-N2-T3-E2-H7
* RVA/Horse-wt/ARG/E403/2006/G14P[12]	G14-P[12]-I2-R2-C2-M3-A10-N2-T3-E12-H7
* RVA/Horse-wt/ARG/E4040/2008/G14P[12]	G14-P[12]-I2-R2-C2-M3-A10-N2-T3-E12-H7
* RVA/Horse-wt/IRL/04V2024/2004/G14P[12]	G14-P[12]-I2-R2-C2-M3-A10-N2-T3-E2-H7
* RVA/Horse-wt/ZAF/EqRV-SA1/2006/G14P[12]	G14-P[12]-I2-R2-C2-M3-A10-N2-T3-E2-H7

* Reference data from Matthijnssens *et al.*, 2012