

Mutations in the spike gene of porcine epidemic diarrhea virus associated with growth adaptation in vitro and attenuation of virulence in vivo

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Abstract Previously, we have reported that a serial passage of 83P-5 strain of porcine epidemic diarrhea virus (PEDV) in Vero cells resulted in a growth adaptation of the virus in cultured cells at the 22nd passage. In this study, we further maintained the 83P-5 in Vero cells up to the 100th passage and analyzed changes in the spike (S), membrane (M), and nucleocapsid (N) gene sequences and pathogenicity of the virus at the 34th, 61st, and 100th passage levels. Sequence analyses revealed a strong selection for the S gene of 83P-5 in Vero cells, and virtually all mutations occurring at the 34th and 61st passages had been carried over to the 100th-passaged virus. In contrast, the viral M and N genes showed a strong conservation during the serial passage. Pigs experimentally infected with the 34th- or 61st-passaged virus, but not the 100th-passaged virus, exhibited diarrhea, indicating an attenuation of the 83P-5 at the 100th passage. Interestingly, S protein of the attenuated 100th-passaged 83P-5 showed a remarkable sequence similarity to that of previously reported DR-13 strain of attenuated PEDV that also had been established by serial passage in Vero cells. Further studies will be required to define whether the mutations in the S gene of 83P-5 that had been selected and accumulated during the serial passages are indeed the causalities of the growth adaptation in vitro and the attenuation of virulence in vivo.

Keywords Growth adaptation · Mutation · Porcine epidemic diarrhea virus · Serial passage · Spike gene · Virus attenuation

Porcine epidemic diarrhea virus (PEDV), a member of the family *Coronaviridae*, causes acute diarrhea, dehydration, and high mortality in piglets [1, 2], which incurs significant economic losses in European and Asian swine industries [3–6]. PEDV has a single-stranded positive-sense RNA genome of approximately 28 kb in size that encodes four structural proteins (spike [S], envelope, membrane [M], and nucleocapsid [N] protein) and four nonstructural proteins (1a, 1b, 3a, and 3b). Among the viral proteins, the S protein that functions in the receptor binding and virus-cell membrane fusion at the time of virus entry represents an important site for virus neutralization [7–10]. It has been well documented that S proteins of several coronaviruses, such as mouse hepatitis virus (MHV), porcine transmissible gastroenteritis virus (TGEV), avian infectious bronchitis virus (IBV), and human severe acute respiratory syndrome coronavirus, play important roles in virus entry, neutralization, and pathogenicity [11–13]. The M protein of coronavirus is the most abundant component among the viral envelope proteins and plays important roles in virus assembly by interacting with S and N proteins [14–19]. The N protein of coronavirus having a RNA-binding property packages viral genomic RNA into the nucleocapsid of virus particles [20, 21].

In general, live attenuated virus vaccines elicit protective immunity more efficiently than inactivated virus vaccines or subunit vaccines that consist of DNA or recombinant proteins. Likewise, the majority of commercially available PEDV vaccines are live attenuated strains of PEDV that have been established by a growth adaptation of the virus through serial passages in vitro [22, 23]. While serial passages of other coronaviruses also often result in growth adaptation in new host cells and attenuation of virulence in their natural hosts, the molecular basis of virus adaptation and attenuation are not yet elucidated.

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Previously, we have isolated 83P-5 strain of PEDV from the small intestine of a piglet with diarrhea by using Vero cells, and the virus was subsequently subjected to a serial passage in Vero cells [24]. The parent 83P-5 replicated only in Vero cells among various cell lines we tested, whereas the virus at the 22nd passage replicated not only Vero cells but also MA104, MPK, and CPK cell lines. Thus, the serial passage of 83P-5 in Vero cells resulted in the viral adaptation to grow in cultured cells. In this study, to better understand the mechanisms underlying in vitro growth adaptation and in vivo attenuation of PEDV, we further maintained the 83P-5 in Vero cells up to the 100th passage and analyzed changes in the S, M, and N gene sequences and pathogenicity of the virus at the 34th, 61st, and 100th passage levels.

Full-length S, M, and N gene cDNAs were amplified from the parent, the 34th-, 61st-, and 100th-passaged 83P-5 by RT-PCR using the Transcriptor High Fidelity cDNA Synthesis kit (Roche Diagnostics, Switzerland) and the Expand High Fidelity Plus PCR System (Roche Diagnostics) with primers shown in Table 1. The resultant amplicons were gel-purified and cloned into the pCR[®] 4-TOPO[®] vector (Invitrogen Corp., USA) and sequenced in both directions.

The S genes of the parent, the 34th- and 61st-passaged 83P-5 viruses consisted of 4,152 nucleotides encoding 1,383 amino acids, whereas the 100th-passaged virus had an in-frame deletion of three nucleotides at position 455–457 that resulted in a Tyr₁₅₂ deletion (Table 2). Compared to the S gene of the parent 83P-5, the 34th-, 61st-, and 100th-passaged viruses had 6, 10, and 18 nucleotide changes, respectively. These mutations were predicted to change 5, 9, and 13 amino acids in the S protein of the 34th-, 61st-, and 100th-passaged 83P-5, respectively. The high nonsynonymous/synonymous ratio

of nucleotide substitutions (5.00, 9.00, and 5.00 in the 34th-, 61st-, and 100th-passaged 83P-5, respectively) implied a positive selection of the mutations in the S genes of 83P-5 during the consecutive passages in Vero cells.

The S protein of PEDV is a type I membrane glycoprotein composed of a signal peptide, S1 and S2 external domains, a transmembrane domain, and a C-terminal cytoplasmic domain. As shown in Fig. 1, the serial passage of 83P-5 up to the 100th passage was associated with amino acid substitutions in the signal peptide and S1 and S2 external domains, yet no mutation was found in the transmembrane or cytoplasmic domains. Thus, the extracellular region of the S protein of 83P-5 was preferentially subjected to selection pressures for the virus replication in Vero cells. Interestingly, all amino acid changes occurring at the 34th and 61st passages, except for Arg₁₀₈₇Gly change at the 34th passage, were found to be carried over to the 100th passage. This implied that the mutations occurring at the 34th and 61st passages were strongly selected for and accumulated in the viral S gene during the course of consecutive passage. In addition to the mutations carried over from the 34th- and 61st-passaged viruses, the 100th-passaged 83P-5 had five mutations specific to this virus, including a Tyr₁₅₂ deletion. As a result, the 100th-passaged 83P-5 showed mutations in close proximity to each other in the S1 domain (Asn₁₁₂/Ser₁₁₄, Tyr₁₅₂ deletion/Leu₁₅₃, and Lys₃₇₈/Ser₃₇₉) and in the S2 domain (Leu₉₆₃/Thr₉₆₅/His₉₇₃). These mutations occurring in close proximity to each other may influence the structure and/or function of the S protein more strongly than the other sporadic mutations.

Previous studies of the S protein of PEDV have identified neutralizing SS2 (a.a. position 748–755) and SS6 (a.a. position 764–771) epitopes in the S1 domain [10] and 2C10 epitope (a.a. position 1368–1374) in the cytoplasmic

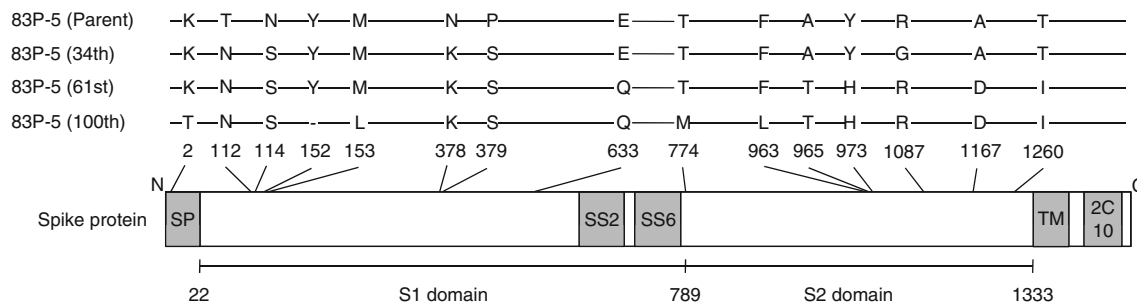
Table 1 Primer sequences for amplification of S, M, and N gene and GenBank accession numbers of each passaged 83P-5 strain

Gene	Primer sequence	Sense	Position in genome ^a	GenBank accession number
S	5'-ATCATTGGTCAACGTAACAA-3'	Forward	20616–20637	AB548618 (parent virus)
	5'-TATTGAAAAAGTCAAGAAACA-3'	Reverse	24811–24790	AB548619 (34th-passaged virus) AB548620 (61st-passaged virus) AB548621 (100th-passaged virus)
M	5'-ACTTGTCACCGTTGTGTAA-3'	Forward	25561–25580	AB618615 (parent virus)
	5'-CCAATTTGCTGGTCTTATT-3'	Reverse	26525–26506	AB618616 (34th-passaged virus) AB618617 (61st-passaged virus) AB618618 (100th-passaged virus)
N	5'-CGGTCAAAACACGGCGACTA-3'	Forward	26279–26298	AB618619 (parent virus)
	5'-CTACCCTGGAACATAGCCA-3'	Reverse	27740–27722	AB618620 (34th-passaged virus) AB618621 (61st-passaged virus) AB618622 (100th-passaged virus)

^a The nucleotide positions correspond to the sequence of PEDV CV777 genome, GenBank accession number AF353511

Table 2 Lengths and mutations in nucleotides and amino acids of the S gene of 83P-5 strain

	Virus			
	Parent	34th-passaged	61st-passaged	100th-passaged
Nucleotides	4152	4152	4152	4149
Amino acids	1383	1383	1383	1382
Nucleotide mutations	–	6	10	18
Amino acid substitutions	–	5	9	13
Synonymous substitutions	–	1	1	3
Nonsynonymous substitutions	–	5	9	15
Asn-Xaa-Ser/Thr sequons	30	29	28	28

**Fig. 1** Schematic representation of mutations in the S protein of the parent, 34th-, 61st-, and 100th-passaged 83P-5. Signal peptide (SP), transmembrane domain (TM), and neutralizing epitopes (SS2, SS6,

and 2C10) are shown as *gray boxes*. Amino acid numbering corresponds to the parent virus. *Dash* indicates deletion

domain [7]. We found no mutations in these neutralizing epitopes during the serial passages of 83P-5 in the Vero cells (Figs. 1, 2).

The S protein of PEDV is heavily glycosylated, and the parent 83P-5 contains 30 potential N-linked glycosylation sites defined by N-{P}-[ST], where {P} is any amino acid except proline. The predicted N-linked glycosylation sites in the S protein of the parent 83P-5 were well conserved in the 34th-, 61st-, and 100th-passaged 83P-5, except for an N-linked glycosylation at Asn₁₁₀ of the parent virus shifted to Asn₁₁₂ in all of the passaged viruses by the close proximal Thr₁₁₂Asn and Asn₁₁₄Ser mutations (Fig. 2). The shift of the N-linked glycosylation site in the close proximity site as well as the conservation of the other N-linked glycosylation sites suggest an importance of the glycan motif in the S protein of PEDV for the protein conformation and/or function, as has been previously shown in S proteins of other coronaviruses [25, 26].

Previously, Park et al. [27] reported the S gene sequences of a live attenuated DR13 vaccine strain of PEDV that was established by a serial passage of the parental virus in Vero cells. To examine whether the 83P-5 and DR13 viruses were subjected to a similar selection pressure during serial passage in Vero cells, the S protein sequences of the parent and the serially passaged 83P-5 were aligned with those of the parent and the attenuated DR13 (Fig. 2). The result revealed a remarkable sequence

similarity between the 100th-passaged 83P-5 and the attenuated DR13. Among fourteen amino acid changes in the 100th-passaged 83P-5 compared to its parental virus, thirteen amino acid changes, including the Tyr₁₅₂ deletion, were also present in the attenuated DR13. The parent strains of 83P-5 and DR13 had forty-seven amino acid differences; among them, only Asn₁₁₂, Ser₃₇₉, and Ala₄₇₄ in the parent DR13 remained in the attenuated DR13. Thus, the S protein of attenuated DR13 appeared to more closely resemble the 83P-5 strains, in particular the 100th-passaged 83P-5, than the parent DR13.

To further define the genetic relationships of the 83P-5 and DR13 parental strains and their serially passaged derivatives, phylogenetic analysis was performed along with full-length S gene sequences of PEDV that were available in GenBank database. The phylogenetic tree topology formed five distinct genetic groups (groups I–V in Fig. 3), and the parent 83P-5 and parent DR13 belonged to the genetically distinct III and IV groups, respectively.

Fig. 2 Alignment of the deduced amino acid sequences of the S protein of the parent and passaged PEDV 83P-5 strains with that of the parent and attenuated DR13 strains [27]. *Dots* represent amino acids that are identical to those in the parent 83P-5. Potential N-linked glycosylation sites of N-{P}-[ST] are *underlined*. *Boxes* indicate the signal peptide (position 1–21), transmembrane domain (1334–1356), and neutralizing epitopes (748–755; SS2, 764–771; SS6 and 1368–1374; 2C10)

	Signal peptide	
83P-5 (Parent)	MKPLIYFWLFLPVLLTSLSPQDVTRCQSTINFRFRFFSKFNVAQPAVVVLLGGYLPMSNSSSWYCGTGIETDSGVHGIFLSYIDSGQGFEIGISQEPFDPDSGYQLYLHKATNGCTNIAIARLR	120
83P-5 (34th)	120
83P-5 (61st)	120
83P-5 (100th)	120
DR13 (Parent)	.RS.....L...P.....T.....A.....N.....	120
DR13 (Attenuated)	.T.....	120
83P-5 (Parent)	ICQFPDNKTLGPTVNDVTTGRNCLFNKAI PAYMQDGKNI VVGITWDRVTVFADKIYHFYIKNDWSRVATRCYNKRSCAMQYVYTPTYMNLNVT SAGEDGIYYEPC TANC SGYAA NVFA	240
83P-5 (34th)	240
83P-5 (61st)	240
83P-5 (100th)-L.....	239
DR13 (Parent)R...D.....L.....R.....T.....	240
DR13 (Attenuated)-L...T.....	239
83P-5 (Parent)	TDSNGHIEPEGFSFNWELLSNDSTLLHGKVVSNQPLLWCLWAIPKIYGLQFFSFNQTMDGVCNGAAQAPEALRFNINDTFVILAEGSVLVLHTALGTNL SFVCSNSSDPHKAIFTIP	360
83P-5 (34th)	360
83P-5 (61st)	360
83P-5 (100th)L.....H.....VD.....S.....L...A...	359
DR13 (Parent)L.....H.....VD.....S.....L...A...	360
DR13 (Attenuated)I.....	359
83P-5 (Parent)	LGVTEVPYCYFLKVDYNTPTVYKFLAVLPPTVKEIVITRYGDVYVNGFGYLHLGLLDAVITNFTGHGTDDDVS GFWT VASTNFVDALIEVQGTAIQRILYCDPVSQKCSQV SFDLDDG	480
83P-5 (34th)KS.....	480
83P-5 (61st)KS.....	480
83P-5 (100th)KS.....	479
DR13 (Parent)A.....S.....R.....I.....S.....A.....	480
DR13 (Attenuated)KS.....	479
83P-5 (Parent)	FYPISRRNLLSHEQPISFVTLPSFNDHSFVNITVSAAFGGHSGANLIASDITTINGFSSFCVDTRQPTITL FYNV TNSYGYVSKSQDNCNPPTLQSVNDYLSFSKFCVSTSLLAGACTIDL	600
83P-5 (34th)	600
83P-5 (61st)	600
83P-5 (100th)L.S...V.....	599
DR13 (Parent)L.S...V.....	600
DR13 (Attenuated)	599
83P-5 (Parent)	FGYPEFGSGVKFTSLYFQFTKGLITGTPKPLEGVTDV SFMTLDVCTRYTIYGFKEGIIITLNS SFLAGVYVTS DSGQLLAFK NVTSGAVYSVT PCSFSEQAAYVDDDIVGVISSLNS	720
83P-5 (34th)	720
83P-5 (61st)Q.....	720
83P-5 (100th)Q.....	719
DR13 (Parent)A.....L.....I.....I.....N.....	720
DR13 (Attenuated)S.....Q.....	719
83P-5 (Parent)	TFNNTRELPGFFYHSNDGSNCTEPVLYSNI GVCRSGSIGYVFLDQGQVKIAPT VTGNIS IPTNFSMSIRTEYLQLYNTPVSVDCVTV CNGNSRCKQLLTQYTAACKTIESALQLSARL	840
83P-5 (34th)	840
83P-5 (61st)	840
83P-5 (100th)M.....	839
DR13 (Parent)P.Y.....A.....	840
DR13 (Attenuated)K.....M.....	839
83P-5 (Parent)	ESVEVNSMLTISEEALQLATISSFN G DGYNFTNVLGVSVDY PASGRVQKR SFIEDLLFNKVVTNGLGTVDEDIYKRC SNGRSVADLVCAQYYS GVMVLPGVVDAEKLHMY SASLIGGMAL	960
83P-5 (34th)	960
83P-5 (61st)	960
83P-5 (100th)A.....V.....	959
DR13 (Parent)A.....V.....	960
DR13 (Attenuated)G.....	959
83P-5 (Parent)	GGFTAAAALFFSYAVQARLNLYALQTDVLRNQQLLAESFN SAIGNITSAFESVKEAISQTSNGLNVTVAHALTKVQEVVNSQGSALTQLT IQLQHNFOAISSSIDDIYSRLDLSADVQV	1080
83P-5 (34th)	1080
83P-5 (61st)T.....H.....	1080
83P-5 (100th)L.T.....H.....	1079
DR13 (Parent)I.....K.....N...V.....	1080
DR13 (Attenuated)L.S...S.H.....	1079
83P-5 (Parent)	DRLITGRLSALNFAVQTLTKYTEVQASRKL AQQKVNCEVKSQSQRYGFCGGDGEHIFSLVQAAPQGLLFLHTVLVPGDFVNVIAIAGLCVNGDIALTLREPGLVLF THELQTYTATEYF	1200
83P-5 (34th)G.....	1200
83P-5 (61st)D.....	1200
83P-5 (100th)D.....	1199
DR13 (Parent)L.....E.....N.....	1200
DR13 (Attenuated)D.....	1199
83P-5 (Parent)	VSSRRMEPRKPTVSDVFQIESCVVTVYNLTSDQLPDVIPDYIDV NKT LDEILASLPNRTGPSLPLDVF NATYLNLTGETADLEQRSELRNTEELRSLIYININNLVDLEWLN RVETY	1320
83P-5 (34th)	1320
83P-5 (61st)I.....	1320
83P-5 (100th)I.....	1319
DR13 (Parent)N.....N.....	1320
DR13 (Attenuated)I.....S.....	1319
83P-5 (Parent)	IKWPWWVLIIFVLIIPVVSLLVFCCISTGCCGCCGCCGACFGCCCGPRLQPYEAFKVVHQ	1383
83P-5 (34th)	1383
83P-5 (61st)	1383
83P-5 (100th)	1382
DR13 (Parent)	1383
DR13 (Attenuated)	1382

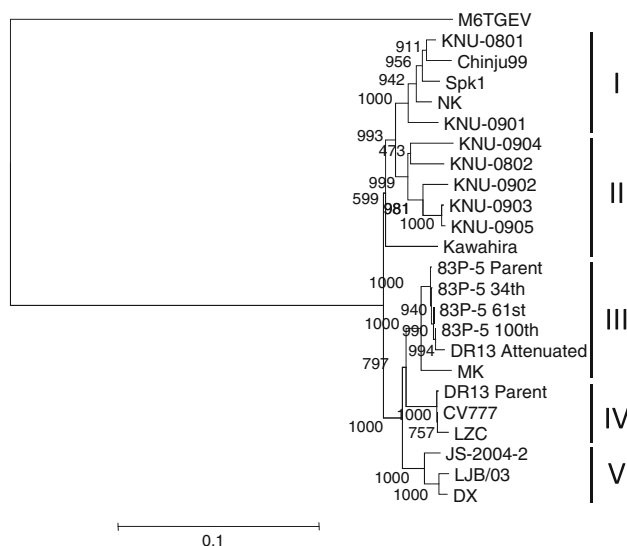


Fig. 3 Phylogenetic analysis of the full-length S gene sequence of PEDV. Neighborhood-joining tree was generated with 1,000 bootstrap replications using Clustal X v1.83 [39] and viewed by MEGA v4.0.2. The phylogenetic tree was rooted with an out-group TGEV strain (accession number 811785). GenBank accession numbers of reference PEDVs for the S gene are AY167585 (Chinju99), AF353511 (CV777), DQ462404 (DR13 attenuated), DQ862099 (DR13 parent), EU031893 (DX), AY653204 (JS-2004-2), AB548622 (Kawahira), GU180142 (KNU-0801), GU180143 (KNU-0802), GU180144 (KNU-0901), GU180145 (KNU-0902), GU180146 (KNU-0903), GU180147 (KNU-0904), GU190148 (KNU-0905), DQ985739 (LJB/03), EF185992 (LZC), AB548624 (MK), AB548623 (NK), and AF500215 (Spk1)

In respect to the 83P-5 strains, the parent, the 34th-, 61st-, and 100th-passaged viruses were clustered into the group III and ranked in order of their passage levels. As predicted, the attenuated DR13 belonged to the group III and was most closely related to the 100th-passaged 83P-5.

We had also cloned and sequenced the M and N genes of each passaged 83-5 strains. The M genes of the parent, the 34th-, 61st-, and 100th-passaged 83P-5 viruses consisted of 681 nucleotides encoding 226 amino acids. Compared to the M gene of the parent 83P-5, 61st- and 100th-passaged viruses had one A to G nucleotide change at position 499 that substituted Asn167 with Asp in an amphipathic domain of the M protein (data not shown), whereas the 34th-passaged virus had no nucleotide change. In the N genes constituting of 1,326 nucleotides encoding 441 amino acids, one silent mutation was found at position 102 of the 100th-passaged virus (data not shown).

It has been shown that a serial passage of PEDV and other coronaviruses often results in attenuation of virus virulence. To investigate if the serial passages of 83P-5 had influenced the viral pathogenicity, PEDV-seronegative 12-week-old pigs were inoculated orally with the 34th-, 61st-, or 100th-passaged viruses. All pigs that were inoculated with the 34th- or 61st-passaged 83P-5 ($n = 3$ each)

showed a watery diarrhea or loose stool by 5 days post-infection (Table 3). In contrast, none of the pigs infected with the 100th-passaged 83P-5 ($n = 4$) showed diarrhea within 14 days post-infection, indicating that the 83P-5 virus had been attenuated somewhere between the 61st and 100th passage levels.

We collected fecal swab samples daily from the infected pigs to analyze virus excretion by nested RT-PCR. The first RT-PCR was performed using the Titan One Tube RT-PCR kit (Roche Diagnostics) with a set of the S gene primers: 5'-TTCTGAGTCAACGACAGCCA-3' (forward) and 5'-CATATGCAGCCTGCTCT GAA-3' (reverse). The second PCR using BioTherm Taq DNA Polymerase (GeneCraft, Germany) was performed with a forward primer 5'-TATTACTGTCTCTGCGGCTTT-3' and the reverse primer of the first RT-PCR. The resultant amplicons were visualized by ethidium bromide staining after electrophoresis in 1.5% agarose gels. Although one out of four pigs infected with the 100th-passaged 83P-5 did not show fecal virus excretion, all other pigs, regardless of the virus inoculum, showed sporadic virus excretion in the feces. Since the PCR analysis of virus excretion is not quantitative, it remains to be determined if there is a significant difference in the virus replication in vivo between the 34th-, 61st-, and 100th-passaged viruses.

In summary, the serial passage of 83P-5 in Vero cells resulted in the viral growth adaptation in vitro and attenuation of virulence in vivo, and these phenotypic changes were associated with a strong selection for the viral S gene. One-directional and cumulative selective pressure for the S gene of 83P-5 in Vero cells was evident by the fact that virtually almost all mutations occurring at early and intermediate passages had been carried over to the 100th-passaged attenuated virus. Given the significance of the S protein for PEDV entry and pathogenicity, the mutation accumulated in the S gene of 83P-5 during the serial passage may contribute to the phenotypic change of the virus. For example, the growth adaptation of 83P-5 in Vero cells had occurred as early as 22nd passage, so that four amino acid changes (Asn₁₁₂, Ser₁₁₄, Lys₃₇₈, and Ser₃₇₉) in the S gene at the 34th passage that were varied over through 100th passage may be responsible for the in vitro adaptation. Similarly, the attenuation of 83P-5 at 100th, but not 61st, passage may be driven by newly acquired five amino acid changes (Thr₂, Tyr₁₅₂ deletion, Leu₁₅₃, Met₇₇₄, and Leu₉₆₃) in the S gene of the 100th-passaged virus.

The remarkable similarity of the S genes between the 100th-passaged 83P-5 and the attenuated DR13 is unusual with respect to previous studies of coronavirus attenuation. Analyses of various IBV strains attenuated in embryonic eggs have shown no common mutations in the S gene [28–31]. In two attenuated swine TGEV strains that were established from different virus strains, only one common amino acid change

Table 3 Positive pigs with diarrhea and viral RNA in fecal swab samples

Group (inoculated virus)	Pig no.		Days post inoculation														
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Group 1 (34th-passaged)	11	Diarrhea ^a	–	–	–	–	+	++	–	–	–	–	–	–	–	–	–
		Viral RNA ^b	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
	31	Diarrhea ^a	–	–	–	–	–	++	–	–	–	–	–	–	–	–	–
		Viral RNA ^b	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
	34	Diarrhea ^a	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
		Viral RNA ^b	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–
Group 2 (61st-passaged)	9	Diarrhea ^a	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–
		Viral RNA ^b	–	–	–	+	–	–	+	+	+	–	–	–	–	–	–
	10	Diarrhea ^a	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
		Viral RNA ^b	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–
	30	Diarrhea ^a	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
		Viral RNA ^b	–	+	+	–	–	–	–	–	+	–	–	–	–	–	–
Group 3 (100th-passaged)	22	Diarrhea ^a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		Viral RNA ^b	–	–	–	+	–	–	–	+	–	+	–	–	–	–	–
	23	Diarrhea ^a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		Viral RNA ^b	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
	24	Diarrhea ^a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		Viral RNA ^b	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
25	Diarrhea ^a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
	Viral RNA ^b	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	

Serially passaged 83P-5 was orally inoculated at $10^{8.2}$ TCID₅₀/pig at day 0

^a Normal stool (–), loose stool (+), diarrhea (++)

^b Positive (+) or negative (–) results for nested RT-PCR using the specific primers to the S gene of PEDV

was found in the S gene [32]. The evolution of the closely related 100th-passaged 83P-5 and attenuated DR13 may be explained by a strong selection pressure on the S gene of PEDV during serial passages in Vero cells, while there should be many other pathways leading to attenuation of coronaviruses.

The 100th-passaged 83P-5 has been licensed for use as an attenuated PEDV vaccine in Japan (Nisseiken Co. Ltd., Japan). As the first step to better understand the mechanisms underlying the attenuation of 83P-5, this study documented the positive selection and accumulation of mutations in the viral S gene during the serial passages in Vero cells. The extensive mutations of the S gene were a contrast to the strong conservation of the M and N genes of 83P-5 during the serial passage. While the single substitution of Asn₁₆₇ with Asp found in the M protein of the 61st- and 100th-passaged virus may influence the virus phenotypes, the influence is likely to be a lesser extent than the extensive S gene mutation does.

Data indicating pivotal roles of S protein in determining phenotypes of coronaviruses have been accumulated, however, it remains to be determined whether the mutations in the S gene of 83P-5 actually contribute to the viral attenuation. In fact, it has recently been shown that

mutations in the non-structural protein of MHV [33], severe acute respiratory syndrome coronavirus [34], and IBV [35] play an important role in the viral attenuation. Furthermore, a deletion of group-specific accessory genes has been shown to confer attenuation of feline infectious peritonitis virus [36], MHV [37], and TGEV [38]. Thus, a mutation in any gene of the ~30 kb genome of coronaviruses that affects virus life cycle in natural hosts potentially results in attenuation of pathogenicity. Further studies will be needed to define the genetic mechanisms that underlie the attenuation of PEDV and other coronaviruses.

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