

Generation and molecular characteristics of a highly attenuated GPV strain through adaptation in GEF cells

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Abstract

Background: Goose parvoviruses (GPVs) spread globally and cause a huge economic loss to poultry industry. Although the attenuated GPV vaccines play a key role in preventing the disease caused by GPV, the molecular basis for the attenuation of GPV is barely known.

Results: A highly attenuated GPV strain, GPV-CZM-142, was generated through blindly passaging of the highly pathogenic strain, GPV-CZM, in goose embryonic fibroblasts (GEF) for 142 generations. The virulence of the GEF-adapted GPV strain was 10000 times weaker than that of its wild type counterpart, GPV-CZM, based on the ELD₅₀. By comparing with the wild type strain, genome sequencing analysis identified a novel insertion in ITR and several other mutations in both NS and VP1 of GPV-CZM-142.

Conclusions: The highly attenuated GPV strain, GPV-CZM-142, provides a GPV vaccine candidate, and the identified virulence-related insertion/mutations give a novel insight into the molecular determinants of GPV virulence.

Background

Goose parvovirus (GPV) belongs to the genus *parvoviruses*, family *Parvoviridae*. GPV is a non-enveloped and single-stranded DNA virus. The genome of GPV contains two inverted terminal repeats (ITRs) and two major open reading frames (ORFs). ITR carries important elements for viral replication and encapsidation. The two major ORFs encode the non-structural protein (NS) and the capsid proteins (VP1/2/3). VP1 is the largest capsid protein, which shares common region of C-terminus with VP2 and VP3. As a globally spread virus, GPV was first isolated and identified in 1956 by Fang in China [1, 2], and its infection mainly causes acute contagious and septic diseases in goslings and muscovy ducks with age younger than 30 days old [3]. The intestinal suppository is a characteristic lesion for GPV infection in goslings[4]. Although the attenuated GPV vaccine and its yolk antibody play a key role in preventing the deterioration of disease caused by GPV, GPV is still frequently isolated in the GPV-vaccinated goose. Also, the current strategies for GPV prevention and control are challenged with the following problems: later onset of GPV infection in goose/duck individuals, more emergence of the GPV mutants, and the co-infection of GPV with other pathogens (e.g., goose astrovirus). However, the molecular basis for virulence of GPV remains unclear. In this study, a highly pathogenic GPV strain, GPV-CZM, was continuously passaged in the GEF for 142 generations to generate a highly attenuated GPV strain, GPV-CZM-142, followed by identification mutations responsible for the attenuation by genomic sequencing analysis.

Methods

Virus strains and goose embryos

A highly pathogenic GPV strain, GPV-CZM, was kept in our laboratory. from 11-day-old goose embryos without GPV maternal antibody, where the goose embryonic fibroblasts (GEFs) were isolated, were kindly provided by Dr. Zhang (Sinopharm Yangzhou VAC Biological Engineering Co. Ltd) .

Preparation of GEFs

A number of 11-day-old goose embryos were selected for GEF preparation. The heads, limbs and internal organs were first removed. The remaining embryo bodies were then rinsed with PBS, followed by digestion with trypsin. After filtering with 6 layers of gauze, the GEFs were cultured in DMEM (Gibco, NY, USA) supplemented with 10% FBS (fetal bovine serum) (Lonsera, Shanghai, China) at 37 °C with 5% CO₂.

Virus passaging

The GEFs prepared above were infected with the GPV-CZM strain (1:10 dilution in PBS) for 2 h. After rinsed with PBS once, the infected GEFs were cultured in DMEM supplemented with 1% FBS for 5-7 days, followed by collection. The cells were then frozen and thaw once. The supernatants were isolated from the infected cells, followed by infecting newly isolated GEFs with it. Like this, the passaging of the virus went into next cycle.

TCID₅₀ assay

GEFs were infected with the viruses that were diluted with DMEM containing 1% FBS at 1:10, 1:10², 1:10³, 1:10⁴, 1:10⁵ and 1:10⁶ in 96-well plates (6 wells per dilution). The cells at day 5 post inoculation were fixed by the ice-cold acetone:ethanol (3:2) mixture for 5 min and washed with PBS. The cells were then stained with the diluted monoclonal primary antibody specific to GPV for 45 min at 37°C. After washing three times with PBS, the cells were stained with the diluted secondary antibody (goat anti mouse IgG-FITC) for another 45 min at 37°C. Again, after three washes with PBS, the cells were observed under an inverted fluorescence microscopy. The TCID₅₀ of these viruses were calculated by the Reed-Muench method based on indirect immunofluorescence intensity.

ELD₅₀ assay

Different GPV strains were diluted with PBS at 1:10, 1: 10², 1: 10³, 1: 10⁴ and 1: 10⁵ and injected into the allantoic cavity of 12 day-old goose embryos (5 embryos for each dilution). The morbidity and mortality of the inoculated goose embryos were recorded daily.

Genome sequencing analysis

The viral DNA were isolated from different GPV strains using the DNA extraction kit and store at -20°C. To amplify the whole genome of GPV, the PCR primers designed based on the genome sequence of the standard strain B (U25749) were used (Table 1) [5, 6]. The PCR products were then cloned into pGEM®-T vector (Promega) and transformed into DH5α *E. coli* competent cells. The recombinant plasmids were extracted and sequenced by Huada Gene Technology Co., Ltd.

Table 1. Primers used for GPV DNA amplification

Primer name	Primer sequence (5'-3')	Position of the fragment
F1	TTCAGCTGCTCATTGGAGGGTT	1-203
R1	TTCTCGAGGCGTGGTCAACCTAACA	(4903-5106)
F2	CGCATGCCGCGCGGTCAGCCCAAT	196-1013
R2	TATGTATGCTGCAGTCACGGTCTT	
F3	TAAGACCGTGACTGCAGCATAAC	1009-2051
R3	TTCGCTCGTTCAGGAACGGGCTCTG	
F4	CAGAGCCCGTTCCTGAACGAG	2022-3041
R4	CAGAGCCCGTTCCTGAACGAG	
F5	CGAACCTGTGGCAGCATCTGAAATG	3010-4099
R5	GTCACCTATTTCCTGCTGTAGTGCTG	
F6	CAGCACTACAGCAGGAATAAG	4072-4911
R6	CGCATGCGCGCGGTCAGCCCAATAG	

Identification of sequence mutations

The whole genome sequences from different GPV-CZM strains were aligned and compared using DNASTar Lasergene software. The regions of ITR, NS and VP1 from two replicates of GPV-CZM were further analyzed with GPV reference strains using MEGA6.1 software (Table 2).

Table 2. Detection of the ELD₅₀ of GPV-CZM-70 and GPV-CZM-142

Virus name	Virus titer		
	TCID ₅₀ /ml	ELD ₅₀ /ml	TCID ₅₀ /ELD ₅₀
GPV-CZM	1.58×10 ⁵	1.08×10 ⁵	1.46
GPV-CZM-70	5×10 ⁵	5	10 ⁵
GPV-CZM-142	5×10 ⁵	<5	>10 ⁵

Results

Highly attenuated GPV-CZM-142 generated through adaptation to GEF

To generate an attenuated GPV vaccine candidate, a highly pathogenic GPV strain GPV-CZM was continuously passaged in GEFs underwent a total of 142 generations. To evaluate the pathogenicity of the adapted GPV-CZM, the ELD₅₀ of wild type

GPV-CZM, the 70th generation of GPV-CZM-70 and the 142th generation of GPV-CZM-142 was tested in goose embryos. The mortalities of the goose embryos infected with the wild type GPV-CZM were 100%, 100%, 80%, 60% and 40% at the infection dose of 1:10, 1: 10², 1: 10³, 1: 10⁴ and 1: 10⁵ dilutions, respectively. In contrast, those of the goose embryos infected with GPV-CZM-70 were 20%, 0%, 0%, 0% and 0%, respectively. Notably, GPV-CZM-142 was not lethal to goose embryos at these doses. Based on the viral TCID₅₀ titers of these GPV strains, The ELD₅₀ of GPD-CZM, GPV-CZM-70 and GPV-CZM-142 were 1.46 TCID₅₀, 10⁵ TCID₅₀ and > 10⁵ TCID₅₀, respectively (Table 2). These data clearly demonstrate that the GPV-CZM-70 and GPV-CZM-142 strains are highly attenuated compared with the wild type GPV-CZM.

Sketch on the genome sequences of different GPV strains

To profile sequence mutations in the genome of the GEF adapted GPV-CZM strains, the genomes of the GPV-CZM, GPV-CZM-70 and GPV-CZM-142 strains were amplified using primers listed in Table 1. As shown in Fig. 1, six fragments with the expected sizes were efficiently amplified. These amplicons were cloned into pGEM®-T Easy vector, followed by sequencing the recombinant plasmids. The whole genome sizes of GPV-CZM, GPV-CZM-70 and GPV-CZM-142 were 5106 bp, 5120 bp and 5128 bp, respectively. The sequence of GPV-CZM, GPV-CZM-70 and GPV-CZM-142 had been submitted to the GenBank (the accession number for GPV-CZM, GPV-CZM-70 and GPV-CZM-142 was MT939902, MT939903 and MT939904, respectively). Alignment analysis of the genomes of the adapted GPV-CZM-70 and GPV-CZM-142 against the wild type GPV-CZM identified a novel insertion in the ITR region. Moreover, 101 single nucleotide mutations in the genome of GPV-CZM-142 compared with the genome of GPV-CZM. Among these mutations, 54, 16 and 31 sites were located in the non-coding region, the NS gene and the VP gene, respectively. Genome phylogenetic analysis indicated that GPV-CZM, GPV-CZM-70 and GPV-CZM-142 strains were clustered into the same branch as the strains GDaGPV, SYG61v, GPV-98E and GPV-98D15 (Fig. 2). It is noteworthy that GPV-98D15 is an attenuated GPV through passaging in duck embryo.

Mutations identified in ITR of GPV-CZM-142

Since ITR plays vital roles in gene expression and viral replication, ITR of the adapted GPV-CZM-70 and GPV-CZM-142 were further analyzed. The size of ITR of GPV-CZM, CZM-70 and CZM-142 was 444 bp, 451 bp and 455 bp, respectively. The homology of these ITRs from GPV-CZM, GPV-CZM-70 and GPV-CZM-142 was 95.5-97.1%. Except for several site mutations, the insertions with 7 bp and 11 bp lengths were identified in the ITRs of GPV-CZM-70 and GPV-CZM-142, respectively, compared with that of the wild type GPV-CZM (Fig. 3). Notably, such insertions were occurred at the same position in the genomes of GPV-CZM-70 and GPV-CZM-142 but not in the other GPV strains analyzed, suggesting the insertions have a potential role in the attenuation of the virus.

Mutations identified in NS and VP1 of GPV-CZM-142

Different from the ITR region, no deletion and insertion were identified in the non-structural protein NS and the structural protein VP1 of GPV-CZM-70 and GPV-CZM-142 compared with the wild type strain of

GPV-CZM. However, there were some single nucleotide mutations identified in the protein-encoding genes of the two strains (Table 4 and 5). Compared with GPV-CZM, GPV-CZM-70 had a total of 18 mutations for amino acid with 2 mutations located in NS gene (Table 4) and the other mutations located in VP1 gene (Table 5). In contrast, GPV-CZM-142 carried 33 mutations for amino acid with 8 mutations located in NS gene (Table 4) and the other mutations located in VP1 gene (Table 5). Notably, of these mutations, 7 and 16 mutations in NS and VP1 were common to GPV-CZM-70 and GPV-CZM-142, respectively. In addition, 7 and 6 mutations in NS and VP1 of GPV-CZM-142 were also found in GPV strain SYG61v, an attenuated vaccine strain. These findings suggest that these mutations are involved in host adaptation of the virus.

Table 3. The information for 17 GPV reference strains used in this study

Strain	Genome/bp	Source	Login ID
GPV-98E	5106	Heilongjiang province, China	KT598506
GPV-98D15	5114	Heilongjiang province, China	KT598505
SYG61v	5102	Jiangsu province, China	KC996729
DY16	5046	Jiangsu province, China	MH209633
LH	5047	Jiangsu province, China	KM272560
SH	5106	Shanghai province, China	JF333590
SHFX1201	5050	Shanghai province, China	KC478066
Y	5106	Anhui province, China	KC178571
Yan2	5106	Anhui province, China	KR136258
GDaGPV	5106	Fujian province, China	HQ891825
G7	5106	Fujian province, China	KR029617
FJ	5049	Fujian province, China	KY511292
FJ01	5104	Fujian province, China	KT232256
MDE	5106	Fujian province, China	MF438102
RC16	5046	Sichuan province, China	KY475562
82-0321	5050	Taiwan province, China	EU583390
B	5106	Hungary	U25749

Table 4. Mutations in NS protein of GPV-CZM-70 and GPV-CZM-142

Table 5. Mutations in VP1 protein of GPV-CZM-70 and GPV-CZM-142

Position	GPV-CZM	CZM-70	CZM-142	SYG61v
72	I	V	V	I
149	Q	Q	L	Q
199	E	E	D	E
203	A	A	S	A
501	L	L	P	P
507	R	R	P	P
508	K	E	E	E
573	E	E	A	E
579	T	T	T	E

Position	GPV-CZM	CZM-70	CZM-142	SYG61v
3	T	N	N	T
5	L	V	V	L
89	Q	Q	R	Q
110	D	D	N	D
142	D	N	N	D
146	T	K	K	T
193	T	P	P	P
201	K	K	K	E
217	V	L	L	V
302	I	I	L	I
360	P	A	A	P
381	N	N	K	N
449	S	R	R	S
503	F	V	V	V
516	L	P	P	P
532	K	Q	Q	Q
534	I	M	M	I
537	I	L	L	L
544	S	P	P	S
545	G	G	S	G
546	S	S	T	S
547	T	T	S	T
549	A	A	T	A
555	R	M	M	M
593	D	E	E	D
643	V	V	I	V

Discussion

The live attenuated GPV vaccine plays critical roles in preventing the disease caused by GPV. However, the frequent emergence of the GPV mutants in the vaccinated goose in China is challenging the current GPV controlling strategies, and the molecular basis for the attenuation of the highly pathogenic GPV need to be elucidated. Previous study indicates that the deletion in the ITR may play roles in the attenuation of the GPV through host adaptation, and GPV strains with short ITR are thought to be less pathogenic [6]. In this study, a highly attenuated GPV strain, GPV-CZM-142, were generated through blindly passaging of a highly pathogenic strain GPV-CZM in GEFs. According to the viral titers of TCID₅₀ and ELD₅₀, the virulence of the host adapted GPV-CZM-142 in GEF was more than 10000 times weaker than that of the wild type GPV-CZM. Notably, genome sequencing analysis revealed that except for site mutations through the genome, the insertions with 7bp and 11bp lengths were identified in the ITR of GPV-CZM-70 and GPV-CZM-142, respectively compared with the ITR of the wild type GPV-CZM at the position of 198 bp. The two insertions occurring in the same position in GPV-CZM-70 and GPV-CZM-142 indicate the importance of this region for host adaptation of the virus. Recently, Zhang et al also report several insertions in other positions in the ITR of the attenuated GPV strains through adaptation in GEF and DEF compared with the wild type strain[7]. However, the roles of these insertions including the two insertions identified here in the attenuation of GPV need to be further investigated.

Although no insertion or deletion was found in the coding regions in GPV-CZM-70 and GPV-CZM-142 compared with the wild type strain, a number of mutations were identified in the NS and VP1 protein-encoding genes in the two adapted GPV strains. As a nonstructural protein, NS plays vital roles in initiating the viral replication and inducing apoptosis in the infected cells [8-10]. VP1, one of the major structural proteins, is thought to bind the cell receptor to mediate the infection of GPV, and plays critical roles in the pathogenesis of GPV. Recently, a rescuing assay of the recombinant GPV with different VP1 genes by Wang et al indicates that the chimeric virus with VP1 derived from the virulent strain was pathogenic to goslings whereas that with VP1 derived from the attenuated strain was non-pathogenic to goslings[11]. Liu et al also report that sites of 164 K, 165 K and 167 K of VP1 were vital for the proliferation of GPV in vitro[12]. It is noteworthy that 7 mutations in NS and 16 mutations in VP1 were common to GPV-CZM-70 and GPV-CZM-142 compared with GPV-CZM. Interestingly, 7 mutations in NS and 6 mutations in VP1 of GPV-CZM-142 are also found in an attenuated vaccine strain, SYG61v. All these findings suggest that the mutations may contribute to the attenuation of the adapted GPV strains. Moreover, several mutations, such as K532Q, I534M, I537L, R555M and D594E, in the VP1 found in GPV-CZM-70 and GPV-CZM-142 were located in or very close to the cell receptor binding domain of VP1, implying their host adaptation and potential roles in the attenuation of GPV. Notably, compared with the GPV-CZM-70, GPV-CZM-142 had 6 mutations (Q149L, E199D, A203S, L501P, R507P, E573A) in NS, 9 mutations in VP1 (Q89R, D110N, I302L, N381K, G545S, S546T, T547S, A549T, V643I) and a large insertion in ITR. These additional mutations in NS and VP1, and the large insertion in ITR in the GPV-CZM-142 may contribute to the difference in virulence between GPV-CZM-70 and GPV-CZM-142.

Conclusion

In summary, a novel highly attenuated GPV strain GPV-CZM-142 was generated through serial passages of the virulent strain GPV-CZM in GEF, and a novel insertion and other mutations related to the adaptation were identified in the genome of the GPV-CZM-142. To our knowledge, this is the first demonstration of the insertion of 11 bp in the ITR region in the host adapted GPV strains. The insertion in ITR affecting the activity of the ITR and contributing to the attenuated phenotype of the two adapted strains need to be confirmed by using mini-genome system or by corresponding recombinant viruses.

Abbreviations

GPV: Goose parvovirus; GEF: Goose embryonic fibroblasts; ITR: Inverted terminal repeat; ORF: Open reading frame; NS: Non-structural protein; VP: Capsid protein; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors contributions

HS and AQ conceived and designed the experiments. YJ, HY and LJ performed the experiments. YJ, AQ, KQ, WJ and HS analysed the data. JY, WJ and KQ contributed reagents/materials/analysis tools. HS, YJ and JY contributed to the writing of the manuscript. HS and YJ prepared the figures. All authors read and approved the final manuscript.

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Figures

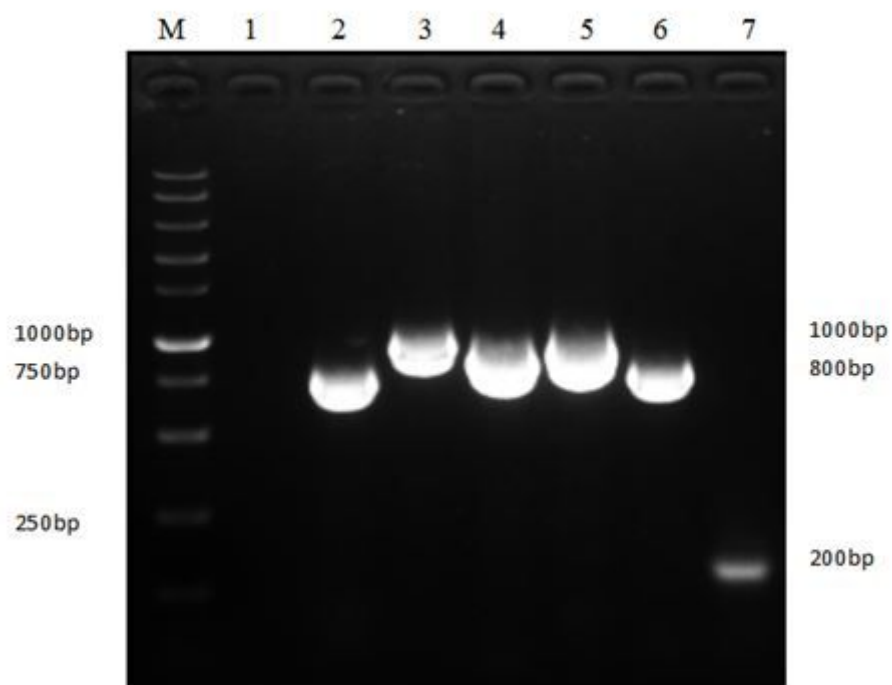


Figure 1

PCR amplification for the genome of GPV Lane M. 1kb Marker; Lane 1. Negative control; Lane 2-7. PCR fragments using primers F2/R2, F3/R3, F4/R4, F5/R5, F6/R6 and F1/R1 respectively.

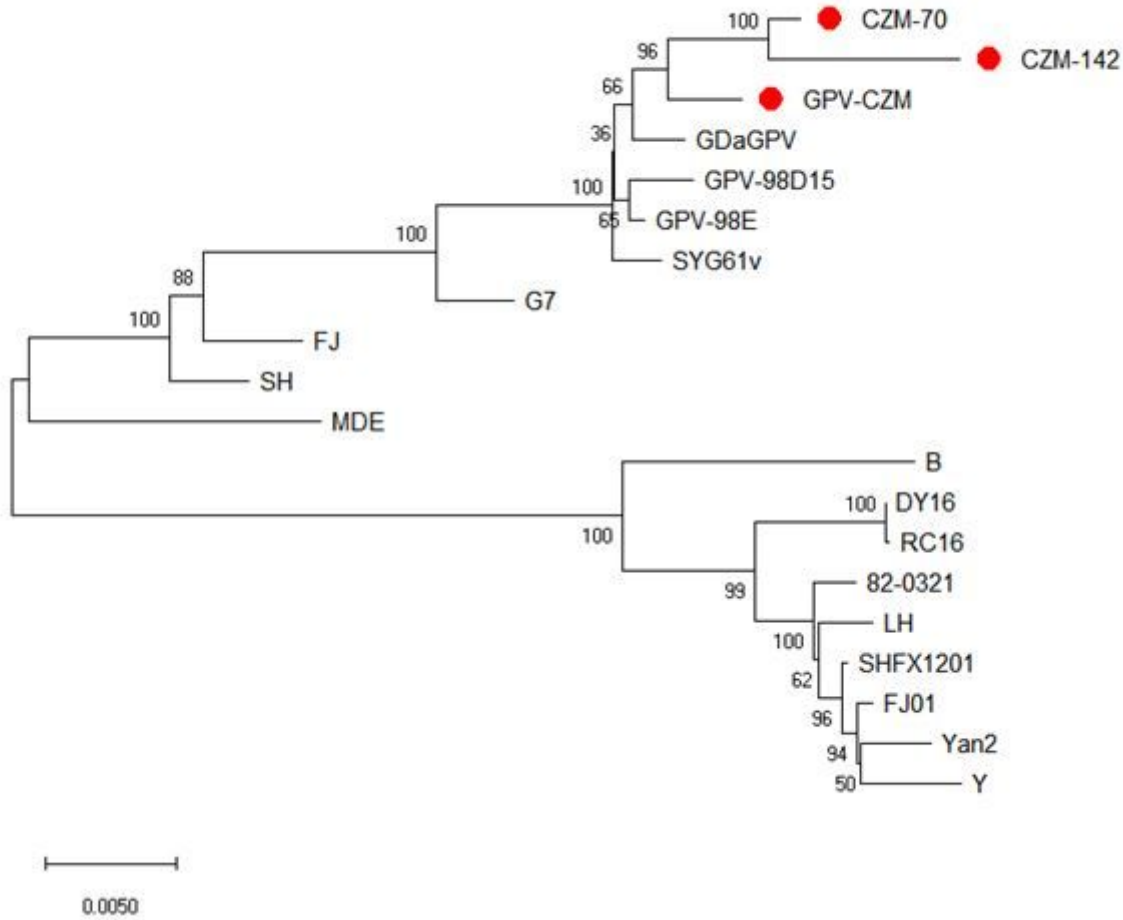


Figure 2

Phylogenetic tree for the genome of GPV-CZM, GPV-CZM-70 and GPV-CZM-142

Majority	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC-----GCATGCCGCGCGGTTCAGCCCAATAGTTAAGCC							
	170	180	190	200	210	220	230	240
CZM-ITR.seq	TCCGGTGACGTATTTCCGGCTGTTAGGTTGACCTCGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATGGTTAATTC			229
CZM-70-ITR.seq	TCCGGTGACGTATTTCCGGCTGTTAGGTTGACCTCGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATGGTTAAGCC			236
CZM-142-ITR.seq	TCCGGTGACGTATTTCCGGCTGTTAGGTTGACCTCGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATGGTTAAGCC			240
B-ITR.seq	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATAGTTAAGCC			229
FJ-ITR.seq	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATAGTTAAGCC			215
Yan2-ITR.seq	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATAGTTAAGCC			229
Y-ITR.seq	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATAGTTAAGCC			229
SHFX1201-ITR.seq	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATAGTTAAGCC			215
RC16-ITR.seq	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGC-GCGCGGTTCAGCCCAATAGTTAAGCC			213
MDE-ITR.seq	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATAGTTAAGCC			229
LH-ITR.seq	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGC-GCGCGGTTCAGCCCAATAGTTAAGCC			213
FJ01-ITR.seq	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAAT-GTTAAGCC			228
DY16-ITR.seq	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGC-GCGCGGTTCAGCCCAATAGTTAAGCC			213
82-0321-ITR.seq	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATAGTTAAGCC			215
SH-ITR.seq	TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATAGTTAAGCC			229
G7-ITR.seq	TCCGGTGACGTATTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATAGTTAAGCC			229
GPV-98E-ITR.seq	TCCGGTGACGTATTTCCGGCTGTTATGTTGACCACAC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATGGTTAAGCC			229
GPV-98D15-ITR.seq	TCCGGTGACGTATTTCCGGCTGTTATGTTGACCACAC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATGGTTAAGCC			229
GDaGPV-ITR.seq	TCCGGTGACGTATTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGCCGCGCGGTTCAGCCCAATAGTTAAGCC			229
SYG61v-ITR.seq	TCCGGTGACGTATTTCCGGCTGTTAGGTTGACCACGC	-----	-----	-----	GCATGC-GCGCGGTTCAGCCCAATGGTTAAGCC			227

Figure 3

Insertions in the ITR region of GPV-CZM-70 and GPV-CZM-142 The ITR region of GPV-CZM, GPV-CZM-70 and GPV-CZM-142 was aligned, and an insertion with 7bp and 11bp length was identified in the ITR of GPV-CZM-70 and GPV-CZM-142 respectively.