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

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Abstract

Background: Goose parvovirus (GPV) has spread globally and causes huge ecomonic loss to poultry industry. Although the attenuated GPV vaccines play vital roles 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 embryo fibroblasts (GEF) for 142 generations. The virulence of the GEF-adapted GPV strain was 10000 times less than that of the wild type GPV-CZM based on the ELD_{50}. Genome sequencing revealed that a novel insertion in ITR and host adapted mutations in NS and VP1 were found in GPV-CZM-142 in comparison with the wild type strain.

Conclusions: The generation of the highly attenuated GPV strain GPV-CZM-142 and the identified virulence-related insertion/mutation profile in the genome not only provided GPV attenuated vaccine candidate, but also gives novel insights into the molecular determinants for GPV attenuation.

Background

Goose parvovirus (GPV) belongs to the genus *parvoviruses*, family *Parvoviridae*. Since its first isolation and identification in 1956 by Fang in China, GPV has spread globally[1, 2]. The infection of GPV mainly causes acute contagious and septic diseases in goslings and muscovy ducks with age in 30 days[3]. The intestinal suppository is the characteristic lesion for GPV infection in goslings[4]. Although the wideusage of the attenuated GPV vaccine and its yolk antibody play vital roles in controlling the deterioration of disease caused by GPV, the frequent isolation of GPV in the vaccinated goose, the delayed age of the GPV onset, the emerging of the GPV mutants and the co-infection of GPV with other pathogens such as goose astrovirus are challenging the current strategies for GPV prevention and control. Moreover, the molecular mechanism for the variation of the field strain and the attenuation of the attenuated vaccine strain is barely known. In this study, a highly pathogenic GPV strain GPV-CZM was serially passaged in the GEF for 142 generations to generate a highly attenuated GPV strain GPV-CZM-142, and a series of mutations in genome related to the attenuation were discovered.

Materials And Methods

Virus strains, cells and goose embryo

A highly pathogenic GPV strain GPV-CZM was kept in our laboratory. The goose embryo fibroblast GEF was prepared by using 11-day-old goose embryos without GPV maternal antibody, which kindly provided by Dr Zhang (Sinopharm Yangzhou VAC Biological Engineering Co.Ltd) .

Preparation of GEF cells

11-day-old goose embryos were selected and the head, limbs and internal organs were first removed. The embryo body was then washed with PBS and digested with trypsin, After filtered with 6 layers of gauze, the GEF cells were cultured in growth medium (DMEM with 10% fetal bovine serum), and incubated at 37 °C with 5% CO2.

Virus passage

The GEF cells prepared above were infected with the GPV-CZM strain (1:10 dilution in PBS) for 2h. After washed with PBS once, the infected GEF cells were cultured in DMEM medium with 1% FBS. The infected cells were cultured for 5-7 days and were freezen and thawn once, and then the supernatants from the infected cells were continued to be passaged to infect the fresh GEF cells.

ELD_{50} assay

Different GPV strains were diluted with PBS in 1:10, 1: 10^2, 1: 10^3, 1: 10^4 and 1: 10^5 dilution and inoculated into 12 Day-old goose embryos (5 embryos for each dilution) through allantoic cavity. The morbility and mortality of the inoculated goose enbryos were monitored daily.

Genome sequencing

The viral DNA from different GPV strains was prepared using the DNA extraction kit, and store at -20℃. For amplifying the whole genome of GPV, the PCR primers referring to the standard strain B (U25749) listed in Table1 were used[5, 6]. The PCR products were then cloned into pGEM®-T vector (Promega) and transformed to DH5α E. coli competent cells. The recombinant plasmids were extracted and sequenced by Huada Gene Technology Co., Ltd.

Sequence analysis

The whole genome sequences of GPV-CZM strain with passage 142 were aligned and compared using DNAStar Lasergene software. The region of ITR, NS and VP1 of two passages of GPV-CZM were further analyzed with GPV reference strains listed in Table2 using MEGA6.1 software.

Results

High attenuation of GPV-CZM-142 through adaptation in GEF

In order to generate an attenuated GPV vaccine candidate, a highly pathogenic GPV strain GPV-CZM was serially passaged in GEF cells. A total of 142 generations of GPV-CZM were generated. To evaluate the pathogenicity of these adapted GPV-CZM in GEF cells, the ELD_{50} of wild type GPV-CZM, the 70th generation GPV-CZM-70 and the 142th generation GPV-CZM-142 was tested in goose embryos. The mortality of the goose embryos infected with the wild type GPV-CZM was 100%, 100%, 80%, 60% and 40% respectively at the infection dose of 1:10, 1: 10^2, 1: 10^3, 1: 10^4 and 1: 10^5 dilutions, whereas that of the goose embryos infected with GPV-CZM-70 was 20% , 0%, 0%, 0% and 0% respectively. Notably, GPV-CZM-

142 was not lethal to goose embryos at all these infection doses tested. Based on the viral $TCID_{50}$ titer of these GPV strains, one ELD_{50} of GPD-CZM, GPV-CZM-70 and GPV-CZM-142 was 1.46 $TCID_{50}$,10^5 $TCID_{50}$ and > 10^5 TCID50 respectively as shown in Table2. This data clearly demonstrate that the GPV-CZM-70 and GPV-CZM-142 strains are highly attenuated in comparison with the wild type GPV-CZM.

Genome amplification and sequencing

To assay the mutation profiles for the GEF adapted GPV-CZM, the genomes of the GPV-CZM, GPV-CZM-70 and GPV-CZM-142 strains were amplified as six fragments using primers listed in Table1. As shown in Fig1, the six fragments with corresponsing sizes could be efficiently amplified. These PCR products were then cloned into pGEM®-T Easy vector and the recombinant plasmids were sequenced. After sequence alignment and joint, the whole genome size of GPV-CZM, GPV-CZM-70 and GPV-CZM-142 was 5106bp, 5120bp and 5128bp, respectively. Genome sequence analysis revealed that the a novel insertion in the ITR region was found in the adapted GPV-CZM-70 and GPV-CZM-142 respectively when compared with the wild type GPV-CZM. Further assay showed that GPV-CZM-142 carried 101mutations for nucleotide in comparison with GPV-CZM. Among these mutations, 54, 16 and 31 sites were located in the non-coding region, the NS gene and the VP genes respectively. Genome phelogenic tree analysis showed that GPV-CZM, GPV-CZM-70 and GPV-CZM-142 strains were clustered into the same branch as strains GDaGPV, SYG61v, GPV-98E and GPV-98D15 as described in Fig2. Among them, GPV-98D15 is an attenuated GPV through passaging in duck embryo.

Insertion and mutation 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 444bp, 451bp and 455bp, 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, an insertion with 7bp and 11bp length was identified in the ITR of GPV-CZM-70 and GPV-CZM-142 respectively in comparison with that of the wild type GPV-CZM as shown in Fig3. Notably, such insertions were occurred at the same position in the GPV-CZM-70 and GPV-CZM-142, which were not found in other GPV strains analyzed, indicating the host adaptation of these insertions and their potential roles in the attenuation.

Mutation sites in NS and VP1 of GPV-CZM-142

Different from the ITR region, no deletion and insertion was found in the non-structural protein NS and the structural protein VP1 of GPV-CZM-70 and GPV-CZM-142 in comparison with GPV-CZM. However, the host adapted mutations were found in both proteins of the two strains as described in Table 4 and 5. Compared with GPV-CZM, GPV-CZM-70 had a total of18 mutations for amino acid. 2 of 18 mutations were located at NS protein (Table 4) whereas other sites were located in VP1 protein (Table 5). GPV-CZM-142 carried33mutations for amino acid in comparison with GPV-CZM. Among them, 8were located in NS protein (Table 4) and 25 were located in VP1 protein (Table 5). Notably, of these mutations, 7 and 16 mutations in NS and VP1 respectively were common in GPV-CZM-70 and GPV-CZM-142 compared with

the wild type GPV-CZM, highlighting the host adaptation of these sites. In addition, 7 and 6 mutations respectively in NS and VP1 of GPV-CZM-142 were also found in GPV strain SYG61v, an attenuated vaccine strain, indicating the roles of these mutations in the viral attenuation.

Discussion

The live attenuated GPV vaccine plays critical roles in preventing the disease caused by GPV. However, the frequent emerging of the GPV mutants in the vaccinated goose in China is challenging the current GPV controlling strategies, and the molecular mechanism for the attenuation of the highly pathogenic GPV need to be elucidated. Previous study indicated that the deletion in the ITR might play roles in the attenuation of the GPV through host adaptation, and GPV strains with short ITR were thought to be low pathogenic or attenuated[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 GEF cells. According to the viral titer of $TCID_{50}$ and ELD_{50}, the virulence of the host adapted GPV-CZM-142 in GEF were more than 10000 times lower than that of the wild type GPV-CZM. Notably, genome sequencing revealed that except for site mutations through the genome, an insertion with 7 bp and 11 bp length was found in the ITR of GPV-CZM-70 and GPV-CZM-142 respectively in comparison with the ITR of the wild type GPV-CZM at the position 198. The two insertions were occurred in the same position in GPV-CZM-70 and GPV-CZM-142 highlights the importance of this region for the host adaptation. Recently, Zhang et al also reported 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 the 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 in comparison with the wild type strain, multiple mutations were occurred in the NS and VP1 protein 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, Wang et al rescued the recombinant GPV with different VP1 genes found 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 reported that sites of 164 K, 165 K and 167 K of VP1 were vital for the proliferation of GPV in vitro[12]. It should be noted that total 7 mutations in NS and 16 mutations in VP1 were commonly found in GPV-CZM-70 and GPV-CZM-142 compared with GPV-CZM. 7 mutations in NS and 6 mutations in VP1 of GPV-CZM-142 were also found in an attenuated vaccine strain, SYG61v. All these indicate that these mutated sites might 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 sited in or very close to the cell receptor binding domain of VP1, indicating their host adaptation and potential roles in the attenuation of GPV.

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 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. Whether the insertion in ITR would affect the activity of the ITR and contribute to the attenuated phenotype of the two adapted strains need to be further elucidated by using mini-genome system or by corresponding recombinant viruses.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

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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Availability of data and materials

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

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Tables

Table 1. Primers used for GPV DNA amplification

Primer name	Primer sequence
F1	TTCAGCTGCTCATTGGAGGGTT
R1	TTCTCGAGGCGTGGTCAACCTAACA
F2	CGCATGCCGCGCGGTCAGCCCAAT
R2	TATGTATGCTGCAGTCACGGTCTT
F3	TAAGACCGTGACTGCAGCATAC
R3	TTCGCTCGTTCAGGAACGGGCTCTG
F4	CAGAGCCCGTTCCTGAACGAG
R4	CAGAGCCCGTTCCTGAACGAG
F5	CGAACCTGTGGCAGCATCTGAAATG
R5	GTCACTTATTCCTGCTGTAGTGCTG
F6	CAGCACTACAGCAGGAATAAG
R6	CGCATGCGCGCGGTCAGCCCAATAG

Table 2. Detection of the ELD_{50} 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	$>10^5$

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

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

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

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

Figures

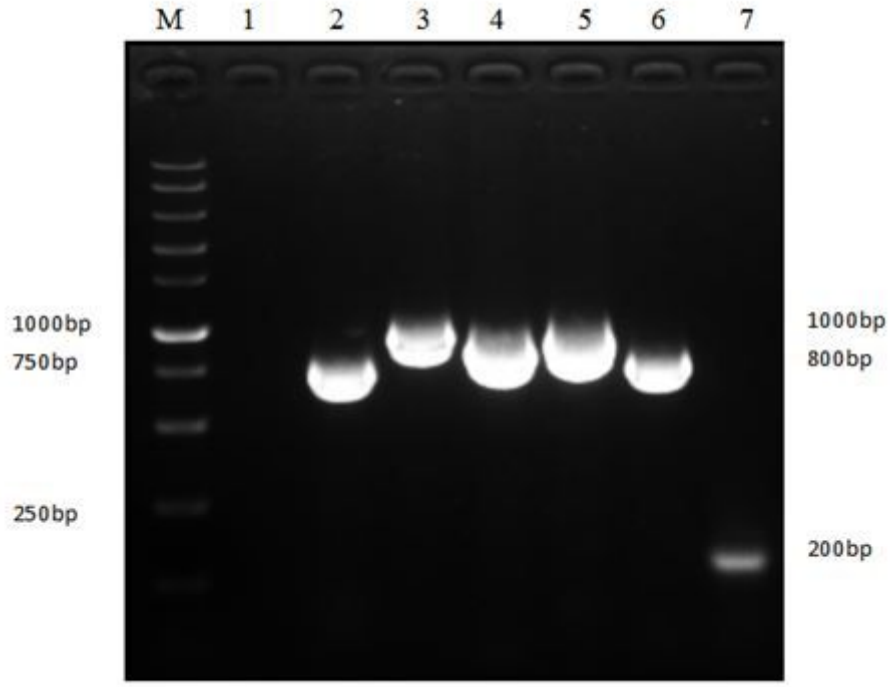

Figure 1

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.

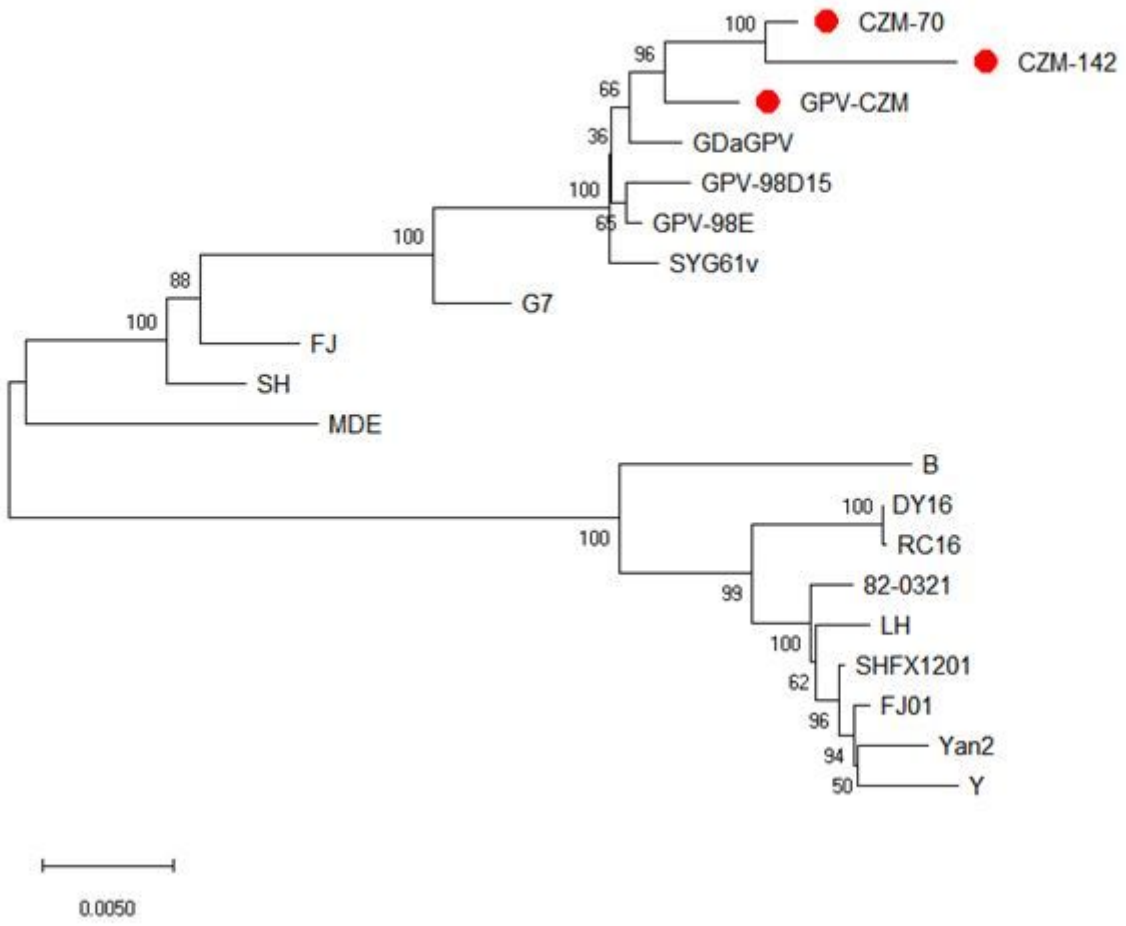

Figure 2

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

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Majority          TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC----------GCATGCCGCGCGGTCAGCCCAATAGTTAAGCC
                            170       180       190       200       210       220       240
CZM-ITR.seq       TCCGGTGACGTATTTCCGGCTGTTAGGTTGACCTCGC----------GCATGCCGCGCGGTCAGCCCAATGGTTAATTC 229
CZM-70-ITR.seq    TCCGGTGACGTATTTCCGGCTGTTAGGTTGACCTCGCC----GGAATCGCATGCCGCGCGGTCAGCCCAATGGTTAAGCC 236
CZM-142-ITR.seq   TCCGGTGACGTATTTCCGGCTGTTAGGTTGACCTCGCCTCGAGGAATCGCATGCCGCGCGGTCAGCCCAATGGTTAAGCC 240
B-ITR.seq         TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC----------GCATGCCGCGCGGTCAGCCCAATAGTTAAGCC 229
FJ-ITR.seq        TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC----------GCATGCCGCGCGGTCAGCCCAATAGTTAAGCC 215
Yan2-ITR.seq      TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC----------GCATGCCGCGCGGTCAGCCCAATAGTTAAGCC 229
Y-ITR.seq         TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC----------GCATGCCGCGCGGTCAGCCCAATAGTTAAGCC 229
SHFX1201-ITR.seq  TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC----------GCATGCCGCGCGGTCAGCCCAATAGTTAAGCC 215
RC16-ITR.seq      TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCGCGC----------GCATGC-GCGCGGTCAGCCCAATAGTTAAGCC 213
MDE-ITR.seq       TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC----------GCATGCCGCGCGGTCAGCCCAATAGTTAAGCC 229
LH-ITR.seq        TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCGCGC----------GCATGC-GCGCGGTTAGCCCAATAGTTAAGCC 213
FJ01-ITR.seq      TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC----------GCATGCCGCGCGGTCAGCCCAAT-GTTAAGCC 228
DY16-ITR.seq      TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCGCGC----------GCATGC-GCGCGGTCAGCCCAATAGTTAAGCC 213
82-0321-ITR.seq   TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC----------GCATGCCGCGCGGTCAGCCCAATAGTTAAGCC 215
SH-ITR.seq        TCCGGTGACGTGTTTCCGGCTGTTAGGTTGACCACGC----------GCATGCCGCGCGGTCAGCCCAATAGTTAAGCC 229
G7-ITR.seq        TCCGGTGACGTATTTCCGGCTGTTAGGTTGACCACGC----------GCATGCCGCGCGGTCAGCCCAATAGTTAAGCC 229
GPV-98E-ITR.seq   TCCGGTGACGTATTTCCGGCTGTTATGTTGACCACAC----------GCATGCCGCGCGGTCAGCCCAATGGTTAAGCC 229
GPV-98D15-ITR.seq TCCGGTGACGTATTTCCGGCTGTTATGTTGACCACAC----------GCATGCCGCGCGGTCAGCCCAATGGTTAAGCC 229
GDaGPV-ITR.seq    TCCGGTGACGTATTTCCGGCTGTTAGGTTGACCACGC----------GCATGCCGCGCGGTCAGCCCAATAGTTAAGCC 229
SYG61v-ITR.seq    TCCGGTGACGTATTTCCGGCTGTTAGGTTGACCGCTG----------GCATGG-GCGCGGTCAGCCCAATGGTTAAGCC 227
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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.