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1	Rapid detection and grouping of porcine bocaviruses by an EvaGreen® based
2	multiplex real-time PCR assay using melting curve analysis
3	
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1 Abstract

2 Several novel porcine bocaviruses (PBoVs) have been identified in pigs in recent 3 years and association of these viruses with respiratory signs or diarrhea has been 4 suggested. In this study, an EvaGreen®-based multiplex real-time PCR (EG-mPCR) 5 with melting curve analysis was developed for simultaneous detection and grouping 6 of novel PBoVs into the same genogroups G1, G2 and G3. Each target produced a 7 specific amplicon with a melting peak of 81.3 ± 0.34 °C for PBoV G1, 78.2 ± 0.37 °C 8 for PBoV G2, and $85.0 \pm 0.29^{\circ}$ C for PBoV G3. Non-specific reactions were not 9 observed when other pig viruses were used to assess the EG-mPCR assay. The 10 sensitivity of the EG-mPCR assay using purified plasmid constructs containing the 11 specific viral target fragments was 100 copies for PBoV G1, 50 for PBoV G2 and 100 12 for PBoV G3. The assay is able to detect and distinguish three PBoV groups with 13 intra-assay and inter-assay variations ranging from 0.13 to 1.59%. The newly 14 established EG-mPCR assay was validated with 227 field samples from pigs. PBoV 15 G1, G2 and G3 was detected in 15.0%, 25.1% and 41.9% of the investigated samples 16 and coinfections of two or three PBoV groups were also detected in 25.1% of the 17 cases, indicating that all PBoV groups are prevalent in Chinese pigs. The agreement 18 of the EG-mPCR assay with an EvaGreen-based singleplex real-time PCR (EG-sPCR) 19 assay was 99.1%. This EG-mPCR will serve as a rapid, sensitive, reliable and cost 20 effective alternative for routine surveillance testing of multiple PBoVs in pigs and 21 will enhance our understanding of the epidemiological features and possible also 22 pathogenetic changes associated with these viruses in pigs.

1

- 2 Keywords: EvaGreen; multiplex real-time PCR; melting curve; porcine bocaviruses;
 3 genogroup; detection
- 4

5 **1. Introduction**

6 Porcine bocavirus (PboV) is a recently discovered virus, which obtained its main 7 name, bocavirus after its first known hosts (bovine and canine) [1, 2]. PBoV belongs to the Bocavirus genus in the Parvovirinae subfamily of Parvoviridae family, which 8 is a group of divergent non-enveloped linear single stranded DNA viruses with a 9 10 genome of approximately 5000 nucleotides comprising an open reading frame (ORF) 11 encoding for non-structural protein NS1 at the 5' end and an ORF at the 3' end encoding for the capsid proteins VP1 and VP2 [3]. Bocavirus, distinguished from 12 other parvoviruses by the presence of an additional third major ORF encoding for 13 NP1 of unknown function located in the middle of the viral genome, is known to 14 15 infect numerous mammalian species including humans, bovine, pigs, gorillas, 16 chimpanzees, California sea lions, dogs, cats, bats and pine martens [4, 5]. Members 17 of this genus, such as bovine parvovirus and canine minute virus which represent two 18 initially identified viruses in this genus, are pathogens that can cause respiratory or 19 enteric disease in their hosts [6]. A recent research strongly supported that the human 20 bocavirus (HBoV) can also be associated with severe acute respiratory tract infection 21 in children in the absence of other viral and bacterial co-infections [7]. The recently 22 discovered novel PBoV was also suggested to be associated with respiratory signs or

- diarrhea, although the pathogenicity of PBoV has not yet been recognized clearly [8,
 9].
- 3

4 Of these bocaviruses, PBoVs exhibit the most genetic diversity [6, 10, 11]. Since the 5 intital discovery of PBoV in Swedish pigs with post-weaning multisystemic wasting 6 syndrome (PMWS) in 2009, a number of additional PBoV has been subsequently 7 discovered and characterized worldwide, and at least 17 novel PBoV species 8 including PBoV1 to PBoV5, PBoV strain WUH1, PBoV H18, PBoV2 A6, PBoV3 22, 9 PboV4 F41, PboV 3C and six newly identified USA strains were identified to date by 10 genome-sequence studies according to the existing criteria for bocavirus classification 11 by the International Committee of Taxonomy of Viruses (http://www.ictvdb.org) [5, 12 11]. Furthermore, mixed infections of a pig with multiple PBoV have been reported in these studies. Thus, it is necessary to develop an effective and accurate approach to 13 14 detect PBoV but also to differentiate PBoV species for epidemiological surveillance and to determine potential associations between PBoV and related diseases. 15

16

17 Although random amplification and large-scale sequencing techniques (viral 18 metagenomic analysis), followed by bioinformatics analysis of large numbers of the 19 sequences of the resulting clones were used in recent years to discover novel PBoVs 20 including the first PBoV [4, 6, 12], these methods are not suitable for epidemiological 21 surveillance on routine sample submissions. Virus isolation combined with electron 22 microscopy or indirect immunofluorescence assay, as a standard laboratory method

1 for diagnosis of viral diseases, was developed to screen pig serum samples for PBoV3 2 and PBoV4, however, this methodology was either not sensitive or specific [13]. The 3 PCR is an alternative rapid virus detection method and several single PCR-based 4 assays have been reported for sensitive and rapid detection of PBoV in clinical 5 samples. However, these methods often just focused on one species or could not cover 6 all the species that have been discovered so far [8-10, 14]. Multiplex methods for the 7 simultaneous detection of several targets offer increased test capacity and reduce 8 overall cost and time, which is desirable for swine disease surveillance. Cai et al. 9 (2013) established a duplex PCR method to simultaneously detect PBoV1, PBoV 2 10 and PBoV3/ PBoV4/ PBoV5, but it was not sensitive and also covered limited species [15]. In order to detect all known PBoV species infecting pigs in clinical samples, a 11 12 multiplex real-time PCR assay has been recently described [5]. This method was specific and sensitive for simultaneous detection and discrimination of all PBoVs that 13 were classified into three groups (PBoV G1, G2, and G3). However, TaqMan probes 14 15 are expensive and time-consuming to synthesize, and high potential false-negative 16 rates have been reported for TaqMan assays due to sequence variability within the 17 probe-binding site [16, 17].

18

In this study, we have developed an EvaGreen®-based multiplex real-time PCR (EG-mPCR) assay followed by melting curve analysis for simultaneous detection of all the different species of PBoV, allowing a rapid, sensitive and specific diagnosis of PBoV infection including of identification of the viral species involved. 1

2 2. Materials and methods

3 2.1. Viruses and samples

PBoV G1 stain MN307 (KF025391), PBoV G2 stain IA147 (KF025392) and PBoV 4 G3 strain IA270 (KF025390) were maintained in the authors' laboratory. To test 5 6 specificity of the assay, the following non-targeted viruses were utilized: transmissible 7 gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) vaccine strain (No. 030718, Harbin Weike Biotechnology development Company, Harbin, 8 9 China), classical swine fever virus (CSFV) (Hangzhou strain), porcine circovirus type 10 1 (PCV1, EF533941), porcine circovirus type 2 (PCV2, GQ996404) and porcine reproductive and respiratory syndrome virus (PRRSV, DQ269472) were maintained in 11 12 the authors' laboratory.

13

A total of 227 pig samples collected from different pig farms in several provinces of 14 15 China during the period from 2013 to 2014 were used in this study. The samples 16 included 200 faecal samples from clinically normal pigs located in the Zhejiang 17 Province and collected during 2013 and 22 healthy serum samples and 5 lung samples 18 from pigs suffered from respiratory tract symptoms and reproductive failure collected 19 from 2013 to 2014. The samples were stored at -80°C until testing. The animal 20 experiments were performed in accordance with international standards for animal 21 welfare and were approved by the Institutional Animal Care and Use Committee of 22 Zhejiang Sci-Tech University.

1

2 2.2. Primers design

3 All of the genomic sequences of the PBoVs utilized in this study were derived from 4 GenBank nucleotide sequence database. The highly conserved regions within each PBoV group genome were aligned with Clustal W (DNAStar Inc., Madison, WI, USA) 5 6 (Fig 1). Primers corresponding to the conserved regions of the viral genomes were 7 designed using Primer Premier 5.0 (Primer Biosoft International, Palo Alto, CA, 8 USA). Three pairs of primers were designed to amplify PBoV G1, G2 and G3 for the 9 conventional PCR and standard plasmid template construction (Table 1). Another 10 three pairs of primers were selected within the range of the amplicons that were 11 capable to amplify and differentiate three PBoV groups with respective distinct 12 amplicon Tm values by melting curve analysis in an EG-mPCR reaction (Table 1). 13 The specificity of the primers was confirmed against random nucleotide sequences obtained by a BLAST search in GenBank databases from the National Center for 14 15 Biotechnology Information (NCBI). All primers were obtained from a commercial 16 source (Sangon Biotech. Co., Ltd, Shanghai, China).

17

18 2.3. Nucleic acid extraction

The samples were processed as described previously [5]. Briefly, tissue samples were minced and diluted 1:10 (w/v) in Dulbecco's modified Eagle's medium, homogenized and centrifuged at 1500 g for 10 min to obtain the supernatant. Faecal samples were resuspended 1:10 (w/v) in PBS, vortexed for 30 s and centrifuged at 1500 g for 10

1 min. Viral genomic DNA was extracted from frozen clinical samples using the AxyPrepTM Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, Hangzhou, China) 2 3 according to the manufacturer's instructions. The extracted DNA was stored at 4 -80°Cu until usage. 5 6 2.4. Plasmid template construction 7 The PCR reactions for PBoV G1, G2 and G3 were conducted in a 25 µL mixture and 8 included 2.5 µL 10× PCR buffer, 1.2 µL 2.5 mM of each dNTPs, 2.5 µL 25 mM MgCl₂, 0.5 µL of each 10 µM primer (Table 1), 1.5 U of Taq DNA polymerase (5 9 10 $U/\mu L$) (Sangon), 2 μL of the DNA and 16 μL distilled water. The amplifications were 11 performed in a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) under the 12 following conditions: after initial denaturation at 95°C for 3 min, 35 cycles were 13 conducted at 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 10 min. The amplicons were detected by electrophoresing 5 µL 14 15 aliquots through 1.5% agarose gel in 1×TAE (40 mM Tris-aceate [pH 8.0], 1 mM 16 EDTA). Each specific viral target fragment was cloned into the plasmid pMD18-T 17 (TaKaRa), and then sequenced by Sangon to construct recombinant standard plasmid 18 templates. 19

20 2.5. EvaGreen®-based multiplex real-time PCR (EG-mPCR) assay to detect and
21 differentiate PBoV G1, G2 and G3

22 To detect and differentiate the DNA of these three PBoV groups in a single step,

8

1	EvaGreen®-based singleplex real-time PCR assays (EG-sPCR) were first developed.
2	Briefly, EG-sPCR for PBoV G1, G2 and G3 were performed in a 10 μL reaction
3	volume containing 1 μL of 10× PCR Buffer and 25 mM MgCl_2, 0.2 mM dNTP mix,
4	0.5 U Taq DNA polymerase (Sangon), 0.5 μL of 20× EG (Biotium, Hayward, CA,
5	USA), 0.2 μ M of the forward and reverse primers (Table 1), and 1 μ L each of plasmid
6	DNA. The amplification was run on an ABI 7300 Detection System (Applied
7	Biosystems, Foster City, CA, USA) under the following conditions: initial
8	denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1
9	min. Based on the established EG-sPCR, a series of experiments were performed to
10	optimize the EG-mPCR protocol, including reagent concentration and PCR cycling
11	parameters. After optimization, the EG-mPCR was carried out in 25 μL of the reaction
12	mixture containing 2.5 μ L of 10× PCR Buffer and 25 mM MgCl ₂ , 0.5 mM dNTP mix,
13	1.5 U Taq DNA polymerase, 1.25 μL of 20× EG, 0.24 (PBoV G1), 0.64 (PBoV G2)
14	and 0.12 (PBoV G3) μ M of each primer pair, and 2 μ L each of plasmid DNA. The
15	amplification and quantification were performed under the following conditions: 5
16	min at 95°C, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 30 s.
17	Melting curve analysis was conducted after each run under the following conditions:
18	The reaction mix was cooled at 60°C for 1 min and then heated at 95°C for 15 s in the
19	ABI 7300 machine. Fluorescence was continuously measured and the melting peaks
20	were calculated by plotting the negative derivative of fluorescence over temperature
21	versus temperature (-dF/dT versus T). The melting peak was defined as melting
22	temperature (Tm value), which was analyzed to distinguish specific amplicons of the

1	three PBoV groups. Amplicons with specific Tm values greater than 77°C and a
2	maximum fluorescence signal over normalized level greater than or equal to 2000 was
3	considered positive.
4	
5	2.6. Detection of three PBoV groups in clinical specimens by EG-mPCR and
6	EG-sPCR
7	A total of 227 clinical specimens from different Chinese pig farms were tested for
8	PBoV G1, G2 and G3 by EG-mPCR and EG-sPCR assays. Each specific viral target
9	fragment was cloned into the plasmid pMD18-T (TaKaRa), and each amplicon was
10	sequenced by Shanghai Sangon Biotechnology Co., Ltd.
11	
12	3. Results
12 13	3. Results3.1. Phylogenetic analysis and primer design of PBoV
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12 13 14 15	 3. Results 3.1. Phylogenetic analysis and primer design of PBoV Initially, available nucleotide sequences of PBoVs were aligned by the Clustal W method and a phylogenetic tree of PBoVs was constructed based on partial and
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12 13 14 15 16 17	 3. Results 3.1. Phylogenetic analysis and primer design of PBoV Initially, available nucleotide sequences of PBoVs were aligned by the Clustal W method and a phylogenetic tree of PBoVs was constructed based on partial and complete or nearly complete genomes. Phylogenetic analysis revealed that these PBoVs including 17 PBoV species clustered into three groups (PBoVG1, G2 and G3)
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12 13 14 15 16 17 18 19 20	3. Results 3.1. Phylogenetic analysis and primer design of PBoV Initially, available nucleotide sequences of PBoVs were aligned by the Clustal W method and a phylogenetic tree of PBoVs was constructed based on partial and complete or nearly complete genomes. Phylogenetic analysis revealed that these PBoVs including 17 PBoV species clustered into three groups (PBoV G1, G2 and G3) (Fig S1). Although the sequences displayed low similarity between groups, the PBoV sequences within each group were found to be relatively conserved (Fig 1), and specific primers targeting each PBoV group were successfully designed for

1 3.2. Specificity detection of PBoV G1, G2 and G3 in the EG-mPCR assay

To determine the specificity of the assay, the three targeted and all non-targeted 2 3 viruses were tested with the EG-mPCR assay. Three discriminated melting peaks for 4 each PBoV group were generated from amplicons after melting curve analysis, while no specific amplification was detected with the other non-targeted pig viruses 5 6 including PCV1, PCV2, PRRSV, CSFV, TGEV and PEDV, although lower melting 7 peaks formed by slight primer dimers were observed (Fig 2A). When all three targeted 8 PBoV groups were tested in the same reaction, three targets were discriminated by 9 three distinct melting peaks through melt curve analysis followed by mPCR 10 amplification, with Tm values of 81.3 ± 0.34 °C for PBoV G1, 78.2 ± 0.37 °C for 11 PBoV G2, and $85.0 \pm 0.29^{\circ}$ C for PBoV G3 (Fig 2B). In addition, the specific 12 amplifications were also confirmed by electrophoresis with a 3% agarose gel (data not shown). These results demonstrated that the EG-mPCR was specific for detection and 13 14 differentiation of the three PBoV groups.

15

16 3.3. Sensitivity and standard curves of the EG-mPCR assay

17 The sensitivity of the EG-mPCR assay was performed by testing serial dilutions of 18 known concentrations of standard plasmid DNAs, and the standard curve was 19 constructed using threshold cycles (C_t) and log inputs for various DNA concentrations 20 ranging from 1.0×10^2 copies/µL to 1.0×10^7 copies/µL with 10-fold serial dilutions to 21 calculate efficiency. The minimum plasmid concentration with a positive result was 22 100 copies/µL (PBoV G1), 50 copies/µL (PBoV G2), and 100 copies/µL (PBoV G3)

1	(Fig 3), and the amplification efficiency of PBoV G1, G2 and G3 was 0.986, 1.027
2	and 1.067 respectively, demonstrating a satisfactory state of amplification. To
3	simulate the infection status of the three PBoV groups in the actual field setting,
4	various template combinations were chosen to determine the sensitivity of the assay.
5	When one PBoV group was present at 1.0×10^6 copies/µL, the detection limit for
6	PBoV G1, PBoV G2 and PBoV G3 was 500, 250 and 250 copies/µL respectively, and
7	250, 250 and 100 copies/ μ L with three PBoV groups mixed in the same
8	concentrations (Fig 3).
9	
10	3.4. Intra and inter-assay reproducibility of the EG-mPCR assay
11	Intra- and inter-test repeatability of the Tm-based method were performed in triplicate
12	for each dilution within the same run and each concentration was repeated at three
13	different times to assess the reproducibility of the EG-mPCR assay. The intra-assay
14	variability for Tm values corresponding to 10^3 - 10^5 copies/µL of each PBoV group was
15	low with a coefficient of variation (CV) from 0.15 to 1.59%. The inter-assay
16	variability of CV for Tm value was also low, in the range of 0.13 to 1.40% (Table 2).
17	Less than 2.0% CV among intra- and inter-tests demonstrated a good repeatability of
18	the assay.
19	Y

20 3.5. Application of the EG-mPCR assay for clinical samples

21 To assess the EG-mPCR for diagnosis of PBoVs, 227 clinical specimens were tested

22 for for all three PBoV groups G1, G2 and G3 by the EG-mPCR and the EG-sPCR

1	using the same three sets of primers (Table 3). Among the 227 clinical samples, 15.0%
2	25.1% and 41.9% were positive for PBoV G1, G2 and G3 by the EG-mPCR. A total
3	of 124 samples were positive with the EG-sPCR. The coincidence between the two
4	diagnostic methods was 99.1%.
5	

In addition, mixed infection of PBoV G1 and G2 was found in 3.1% of the samples, PBoV G1 and G3 in 5.7%, PBoV G2 and G3 in 13.2%, and all three PBoV groups were detected in 3.1% of the 227 samples when tested with the EG-mPCR assay. The positive samples were confirmed by sequencing and all the sequences obtained clustered in their respective groups by phylogenetic analysis. These results indicated that the EG-mPCR could be applied for detection and differentiation of the three PBoV groups in clinical samples and for epidemiological investigation.

13

14 **4. Discussion**

Since its discovery in 2009, PBoV has been detected globally. To date, eleven 15 16 countries have reported infections of PBoV, although the frequency of the reported 17 infections varied from country to country [18-21]. PBoV G1 was found to be almost 18 twice as prevalent in pigs affected by porcine circovirus associated disease (PCVAD) 19 than in non-PCVAD pigs in Sweden from 2003-2007 [22]. A similar trend was also 20 found in Chinese pigs [8, 9], indicating that PBoV G1 might have close relationship 21 with swine respiratory tract diseases, while no significant difference was noted in the 22 detection rate for PBoV G3A and PBoV G3B in fecal or lung samples from healthy

and diseased pigs [14]. Similarly, human BoV1 (HBoV1) is known to be a respiratory
pathogen, while HBoV2-HBoV4 are likely putative agents causing gastroenteritis [23].
The findings in humans suggest that potential associations between PBoVs and related
diseases may exist. It is therefore important to develop an effective and reliable
method to demonstrate multiple PBoV species or genotypes with one single assay for
epidemiological surveillance and disease management of PBoV.

7

In the present study, all currently available partial and complete or nearly complete 8 9 genome sequences that have been deposited in GenBank were aligned and 10 phylogenetic analysis showed that PBoV feld into three distinct genetic lineages, thus 11 generating three bocavirus groups designated PBoV G1, G2 and G3 based on the 12 earliest dates of publications describing the first members of these clusters. This 13 classification is in agreement with previous studies [4, 5, 24]. Despite the high genetic diversity observed in PBoV, the sequences within each group are relatively conserved 14 15 and designing a specific primer targeting each group is possible. The EG-mPCR assay 16 was developed based on the melting curve analysis of different amplicons for each 17 PBoV group using an EvaGreen® dye with a distinct melting temperature (Tm) for 18 each specific melting peak. The final Tm values of the three PBoV groups were $81.3 \pm$ 19 0.34° C, $78.2 \pm 0.37^{\circ}$ C and $85.0 \pm 0.29^{\circ}$ C, respectively, which can be easily 20 differentiated from each other and used for identification of each PBoV group. The 21 assay did not generate any specific melting peak when non targeted pig viruses and a 22 blank control were tested which is suggestive of good specificity. Despite small melt

peaks corresponding to primer dimer observed in this study, which was believed to occur even under optimal conditions regardless of primer design and primer complementarity [25], these small melt peaks could be easily discriminated from the target amplicons because of a lower Tm value and signal strength. Furthermore, sequencing the positive samples confirmed the specificity of the assay.

6

7 High sensitivity is important for diagnostic tools. The EG-mPCR assay described here could detect as few as 100 copies/µL for PBoV G1, 50 copies/µL for PBoV G2 and 8 9 100 copies/µL for PBoV G3, although slight primer dimers were observed in some 10 cases, which may affect the assay sensitivity. The assay was more sensitive than 11 conventional PCR assays which were reported to have a detection limit of around 10^5 copies/µL for PBoV [15], and even compared with TaqMan based real time PCR 12 13 which had detection limit of 600 copies/µL for PBoV2 [5]. Similar sensitivities for EG-mPCR assays were also reported previously for simultaneous detection of six pig 14 15 viruses with detection limits ranging from 100 to 500 copies/µL [26]. Under field 16 conditions, pigs can be co-infected with certain PBoV groups [5]. To account for this, 17 four mixed combinations were chosen to further validate the sensitivity of the 18 EG-mPCR assay. The sensitivity of three PBoV groups decreased with the limits of 19 detection ranging from 250 to 500 copies/µL when combined with one or two groups. It is notable that with one PBoV group present at a fixed concentration of 1.0×10^6 20 21 copies/µL the detection limit for PBoV G1 or G3 was higher than that with three 22 PBoV groups mixed in the same concentrations (Fig 3). This may be caused by

1 multiple targets competing for enzymes and nucleotides, interaction of primers with 2 each other and interference with each other of different melt peaks when 3 co-amplifications are performed in one tube, as was also observed in a previous study 4 [26]. Nevertheless, the assay was still comparable to probe-based multiplex real-time PCRs which had a detection limit around 10^2 copies/ μ L [27, 28]. In addition, the 5 6 EG-mPCR is highly repeatable with both intra-assay and inter-assay variation within 7 2%. These data demonstrated a good specificity, sensitivity, repeatability of the 8 EG-mPCR assay.

9

10 The established EG-mPCR assay was then applied to the detection of PBoV present in 11 clinical samples from pigs. Among 227 clinical samples, a total prevalence of 53.7% 12 was detected for PBoV by the EG-mPCR, of which 99.1% of the positive results were in agreement with the EG-sPCR assay. Among the PBoV groups, PBoV G3 had the 13 highest overall prevalence of 41.9% and PBoV G1 had the lowest overall prevalence 14 15 of 15.0%. The relative ratio of the detection rates for the three PBoV groups was 16 similar to the relative size of three branches in the phylogenetic tree (PBoV G3 17 branch > PBoV G2 branch > PBoV G1 branch), although most of porcine samples 18 examined in this study were from faeces, mainly considering ease if collection of this 19 sample type and a higher assumed prevalence rate in faecal samples compare with 20 other sample types [14]. Our findings were similar to the prevalence of PBoV groups 21 or subgroups ranging from 17.2 to 43.1 % in American pig herds [5]. Interestingly, 22 while a higher prevalence rate of PBoV (81.8%) was detected in serum samples from

1	healthy pigs in this study compared with 21.3% in serum samples from clinical US
2	pigs and 40% in healthy Chinese pigs [29], the former did not detect any PBoV G1,
3	and the latter two just identified PBoV G1 and/or G2. Nevertheless, high prevalent
4	rate of these viruses were detected in this study in porcine faecal, lung, serum samples,
5	although most of which were from porcine faeces which was also the most frequent
6	sample type tested, suggesting a wide tissue tropism of these viruses [5, 10, 14].

7

Lau et al. first reported a Hong Kong pig infected with two subtypes of PBoV G3 8 (PBoV4-1 and PBoV4-2) [14]. Recent research indicated that co-infection with 9 10 multiple different sequences belonging to the same or different PBoV group(s) in the 11 same sample type was a common finding in swine herds in the USA and in China [5, 30]. In the current study, mixed infections of PBoV G1 and G2, PBoV G1 and G3, 12 PBoV G2 and G3 and PBoV G1, G2 and G3 were detected in 3.1%, 5.7%, 13.2% and 13 3.1% of 227 samples, respectively. Recombination, as an integral part of the evolution 14 15 of many viruses, has been reported within the Parvoviridae viral family [31, 32]. The 16 high rate of coinfections of distinct PBoVs in the same sample may indicate ongoing 17 viral transmission from multiple sources and therefore may potentially facilitate 18 recombination and accelerate viral evolution. These findings suggest that these viruses 19 are in the process of adaptation and can undergo rapid evolution to generate new 20 genotypes or species. This is further supported that PBoV have been identified as 21 having the highest genetic diversity among parvoviruses [5, 6, 10].

22

1	In summary, the EG-mPCR assays described here provides an alternative tool for
2	simultaneous, rapid, sensitive and low-cost detection of PBoV G1, G2 and G3 in
3	swine for epidemiological surveillance, which could help to better understand
4	evolutionary characteristics, epidemiology and disease association of PBoVs.
5	
6	Conflict of interest
7	The authors declare that they have no conflict of interest.
8	
9	Acknowledgments
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13	Author Contributions
14	Conceived and designed the experiments: YHJ. Performed the experiments: XWZ
15	GPL ZNW. Analyzed the data: YHJ XWZ TO ZQY. Contributed to the writing of the
16	manuscript: YHJ TO. All authors have approved the present article.
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1 Fig 1 Primer position of three PBoV groups demonstrated by alignment of partial and complete or

- 2 nearly complete PBoV genome sequences available in GenBank. (A) PBoV G1; (B) PBoV G2; (C)
- 3 PBoV G3.
- 4 5

6 Fig 2 Specificity of the EvaGreen® multiplex real-time PCR assay for PBoV demonstrated by 7 melting curve analysis. (A) Specific melting peak was observed for each PBoV group alone, and 8 no specific curve was observed for PCV1, PCV2, CSFV, PRRSV, PEDV and TGEV. (B) Specific 9 melting peaks corresponding to each PBoV group were observed with the three groups present in a 10 single tube at a concentration of 10^6 copies/ μ L.

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13 Fig 3 Sensitivity of the EG-mPCR assay. (A)-(C) Sensitivity for PBoV G1, PBoV G2 and PBoV 14 G3, respectively. (D) Sensitivity for PBoV G1 with a background of 10^6 copies/ μ L PBoV G2. (E) Sensitivity for PBoV G2 with a background of 10⁶ copies/µL PBoV G3. (F) Sensitivity for PBoV 15 G3 with a background of 10^6 copies/uL PBoV G2. (G) Sensitivity for three PBoV groups mixed in 16 17 the same concentrations. Specific melting curve obtained with minimum quantity of standard 18 plasmid DNAs was considered to be the detection limit of the assay.

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21 Fig S1 Phylogenetic analyses of PBoV based on partial and complete or nearly complete genomes 22 available in GenBank. The phylogenetic tree was constructed using MEGA5 software with the 23 maximum-likelihood method under a bootstrap test of 1000 replicates.

Primer	Primer sequence $(5' \rightarrow 3')$	Expected	Position	Reference
		product (bp)		sequence
Primers for co	nventional PCR and standard plasmid construct	tion		
PBoV G1-F	AATACCCATACTCACAAAG	531	3991-4521	HQ291308
PBoV G1-R	GTGATTGATTCATTGCTG			
PBoV G2-F	TCCACTGCTTCGAGAACATC	291	2519-2809	HM053693
PBoV G2-R	TTCCCTGACATCTTTCCATT			
PBoV G3-F	GAAATGTTAGAAGCTGTTGA	384	3098-3481	NC_016031
PBoV G3-R	TACAGGTGACGTTTATTGC			
Primers for the	e EvaGreen® single and multiplex real-time PC	CR assays		
PBoV G1-F	TGAGCTAATCCCTGAACTG	94	4022-4115	HQ291308
PBoV G1-R	GTCTGAGCCTGTATCACCTAT			
PBoV G2-F	GGGCACTGATTATATCTTTAC	86	2722-2807	HM053693
PBoV G2-R	CCCTGACATCTTTCCATT		\mathbf{C}	
PBoV G3-F	ACTCTTTGCAGTCTGACTCTTC	108	3328-3435	NC_016031
PBoV G3-R	GTTCCCCCGTGTCTTTAG			

Table 1. Primer information for the conventional PCR and the EvaGreen® single and multiplex real-time PCR assays for PBoV detection

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PBoV	concentration	Intra-assa	ıy	Inter-as	say	
	(copies/µl)	CV%	SD	CV%	SD	
PBoV	10 ⁵	0.20	0.16	0.27	0.22	
G1	10^{4}	0.37	0.30	0.42	0.34	
	10^{3}	1.59	1.29	1.73	1.40	
PBoV	10 ⁵	0.15	0.12	0.17	0.13	
G2	10^{4}	0.32	0.25	0.43	0.34	
	10^{3}	0.87	0.68	1.45	1.13	
PBoV	10 ⁵	0.20	0.17	0.36	0.31	
G3	10^{4}	0.56	0.48	0.71	0.60	
	10^{3}	0.91	0.77	1.32	1.12	

Table 2. Reproducibility of the EvaGreen multiplex real-time PCR assay

Method	Sample	Health	No.	PBoV G1	PBoV G2	PBoV G3	PBoV G1	PBoV G1	PBoV G2	Three	Total [no.
	Туре	status	samples	only [no.	only	only [no.	+ PBoV	+ PBoV	+ PBoV	PBoV	positive
				positive	[no.	positive	G2 [no.	G3 [no.	G3 [no.	groups	(%)]
				(%)]	positive	(%)]	positive	positive	positive	[no.positi	
					(%)]		(%)]	(%)]	(%)]	ve (%)]	
Multiplex	Faeces	Healthy	200	7(3.5)	11(5.5)	39(19.5)	7(3.5)	13(6.5)	19(9.5)	5(2.5)	101(50.5)
real-time	Serum	Healthy	22	0(0)	2(9.1)	5(22.7)	0(0)	0(0)	9(40.9)	2(9.1)	18(81.8)
PCR	Tissue	Diseased	5	0(0)	0(0)	1(20)	0(0)	0(0)	2(40)	0(0)	3(60)
	Total		227	7(3.1)	13(5.7)	45(19.8)	7(3.1)	13(5.7)	30(13.2)	7(3.1)	122(53.7)
Singleplex	Faeces	Healthy	200	4(2)	11(5.5)	39(19.5)	7(3.5)	11(5.5)	16(8)	15(7.5)	103(51.5)
real-time	Serum	Healthy	22	0 (0)	0(0)	3(13.6)	0(0)	0(0)	13(59.1)	2(9.1)	18(81.8)
PCR	Tissue	Diseased	5	0(0)	0(0))	1(20)	0(0)	0(0)	2(40)	0(0)	3(60)
	Total		227	4(1.8)	11(4.8)	43(18.9)	7(3.1)	11(4.8)	31(13.7)	17(7.5)	124(54.6)

Table 3. Detection of clinical samples by EG-mPCR and EG-sPCR

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ARD A

Fig	g 1													
А	∑ Consensus 62 Sequences	GGGATGCATGCCG 4400	66AGCT6 4410	CCTCACGAGATCT 4420	GGAAGCTACC. 4430	ACAATACGCC 4440	TAC 4450	-ATCACGGTGA	CCTCACAGA- 4470	-CACAAGACAC 4480	4490	4500	GAACT 45	
	HQ223038	TGAGCTAATCCCT	GAACTG	CAGGGATGATT	AT AAACT ACC.	AAACT ATT GC	T AC	TTCCAGGAA	TAGGTGAG	TAGGTGATAC	AGGCTC	- AGAC <mark>ITA</mark> AG	AGAAT	ר ר
	HQ291308	TGAGCTAATCCCT	GAACTG	CAGGGATGATT	AT AAACT ACC.	AAACTATTGC	TAC	TTCCAGGAAG	TAGGTGAC	TAGGTGATAC.	AGGCTC	-AGACTTAAG	AGAAT	
	JX854557 WE025201	TGAGCTAATCCCT	GAACTG	CAGGGATGATTT	AT AAACT ACC.	AAACTATTGC AAACTACTCC	TAC	TTCCAGGAAC	TAGGTGAC TAGGTGAC	AT AGGT GAT AC.	AGGCTC	-AGACITAAG	AGAAT	PRoV CI
	KJ522355	AGAGCTGATACCA	GAACTG	CTGGCACGGTAT	AT AAACT GCC.	AAATT ACT GT	TAC	TTTCAAGAA(TGGGAGAG	ATTGGAGACGG.	AAACCC	-AGAC GTACS	TAACA	
	KJ755665	TGAGCTAATCCCT	GAACTG	CAGGGATGATT	AT AAACT ACC.	AAACTATTGC	TAC	TTCCAGGAA	TAGGTGAG	AT AGGT GACAC.	AGGCTC	-AGACTTAAG	AGAAT	
	KR014255	TGAGCTAATCCCT	GAACTG	CAGGGATGATT	AT AAACT ACC.	AAACT ATT GC	T &C	TTCCAGGAAG	стабетбаф	AT AGGT GACRC.	AGGCTC	-AGAC <mark>ITA</mark> AG	AGAAT	J
	HM053593	GGGGGCGTCACCT	GAGCTT	CC <mark>GA</mark> ACGAGATCT	GGAAGCTGCC	GCAGTACGCC	TACTTCCAG	TATCAGGGAGA	ICT GACCGA	CACGCAACAG	AAATACGCO	ACAG ACGTG	GAAAG	٦
	HMU53594 H0291209	GGGGGGGGTCACCT	GAGETT	CCGAACGAGGTCT CCGAACGAGGTCT	GEARGETACC	GCAGTACGCC GCAGTACGCC	TACTTCCAS	TATCAGGCAGAI	PCTGACCGA	reacersacae	AAACACGGG	ACAGA ACGTO	GABCE CAASC	
	KF025392	AGGGGCGTCGCCC	GAGCTT	CC GA ACGAGATAT	GGAAGCTACC	GCANTACOCC GCANTACOCC	TACTICCAG	TATCAGGGAGA	ICTGACCGA	CACACCACGA	ACCAGACGG	CCAGAACGTG	GAGCG	
	KF025393	GEGEGECETCACCT	GAGCTT	CC <mark>GA</mark> ACGA <mark>A</mark> ATCT	GGAAGCTACC.	ACAATACGCC	TACTTCCAG	TATCA <mark>G</mark> GG <mark>A</mark> GA <mark>T</mark>	rctgaccga	TAACACCACGG	ACAGACGGG	CCAGA ACGT 6	GAGCG	
	KF205155	GGGGGGCATCACCA	GAGCTT	CC <mark>GA</mark> ACGAGATCT	GGAAGCTACC	GCAGTACGCC	TACTTCCAG	T AT C A <mark>g</mark> gg <mark>a</mark> ga <mark>t</mark>	ICTGACCGA	GACGCCACGG	CACATACGGO	CCAGE ACGT 6	GAAC <mark>G</mark>	
	KF205155	AGGGACGTCGCCC	GAGCTT	CCGAACGAGATCT	GGAAGCTACC	GCAGTACGCC	TACTTCCAG	TATCAGGGAGAT	ICTGACCGA	CACACCACGG	AGCCACGGG	CACAGA ACGTO	GAGCG	DD-37.04
	KF205150	GEGEGEGETETEET	GAGCTT	CCGAACGAGATCT	GGAAGCT ACC	GCAGTACGCC GCAGTACGCC	TACTTCCAG	TATCAGGGAGAT	TCTGACCGA	CACGCAACAG	AAATACGCO	ACAGAACGTG	GAAAG	PD0Y G2
	KF206161	GEGEGECETCACCO	GAGCTT	CCGAACGAAATCT	GGAAGCTACC	GCAGTACGCC	TACTTCCAG	TATCAGGGAGA	TCTGACCGA	CACGCAACAG	AAATACGCO	ACAGAACGTG	GAAAG	
	KF205153	Gegegeetcooot	GAGTTT	CC <mark>GA</mark> ACGAG <mark>T</mark> TCT	GGAAGCTACC	GCAATACGCC	TACTTCCAG	T AT C AGGG <mark>A</mark> GAT	ICT GACCGA	CACGCAACAG	AAATACGCO	TACAGA <mark>acgt</mark> g	GAA <mark>AG</mark>	
	KF205155	GGGGGCATCGCCC	GAGCTT	CC <mark>GA</mark> ACGAGATCT	GGAAGCTACC	GCAGTACGCC	TACTTCCAG	TATCAGGGAGAT	ICTGACCGA	CACGCAACAG	CAAATACGCO	CACAGE ACGTG	GAAAG	
	KF206167 KJ755666	GEGEGEGETEACCO	GAGCTT	CCGAACGAGATCT CCGAACGAGATCT	GGAAGCTACC	GCAGTACGCC GCAGTACGCC	TACTTCCAG	TATCAGGGAGAT TATCAAGGAGAT	FCTGACCGA FCTGACCGA	FCACACCACGG	TRACCACAGA	CACAGAACGTG	GAGCG	
	KM402139	AGGAGCGTCACCO	GAGCTT	CCGAACGAGATCT	GGAAGCTACC	GCAGTACGCC	TACTTCCAN	TATCAGGGAGA	TCTGACCGA	GACACCACGG	ACAGACGGG	CCAGAACGTG	GAGCG	
	NC_024453	GEGEGECETCACCT	GAGCTT	CC <mark>GA</mark> ACGAGATCT	GGAAGCT <mark>G</mark> CCI	GCAGTACGCC	TACTTCCAG	T AT C AGGG <mark>A</mark> GAT	TCTGACCGA	CACGCAACAG	AAATACGCO	TACAGA <mark>ACGT</mark> G	GAA <mark>AG</mark>	
	KF205158	AGGATGCATGCCG	GA <mark>AT</mark> TG	CCTCA <mark>T</mark> GAG <mark>GTT</mark> T	GG <mark>GCA</mark> CTACC.	ACAATACGCC	TAC	-ATCACTGTCG	CCTCACA <mark>A</mark> A	-C <mark>G</mark> CAAGACAC	AACCAA1	raate- <mark>ca</mark> gat	GA <mark>GG</mark> T	
	KF205159	AGGATGCATGCCG	GAGTTG	CCTCATGACATCT	GGGAGCTGCC	CAATACGCC	TAC	- ATT AC AAT GCO	CCTCACAAG	-CACAAGATAC.	AGACAA(16AGGAA	GAACG	
	KF206164	AGGATGTATGCCG	БА <mark>А</mark> СТЫ ББА <mark>А</mark> СТЫ	CCTCATGAAGTCT CCTCACGAAGTCT	GEGRACTACC. GEGRACTACC.	ACAATACGCC ACAATACGCT	ТАС ТАС	-CTCACCGTCTC -CTCACCGTGGG	CETCACAAT	-CACAAGATAC	BAAAAAATTTU BAAAAAAATTTU	TECG-GEGAT TAACG-GEGAE	GAGGT	1
	KF205155	GGGATGCATGCCG	GAACTG	CCTCACGATATCT	GGAAACTGCA	GCAATACGCC	TAC	-ATCACGGTCGT	rcecteeee	-CGCAGGACTC	GTAAA	AACG- <mark>GA</mark> GAC	GAGCT	
	KF205158	GGGCTGTATGCC2	GAGCT A	CC <mark>A</mark> CA <mark>T</mark> GA <mark>AG</mark> TCT	GG <mark>GCC</mark> CTAC <mark>A</mark> .	ACAAT ACGC <mark>T</mark>	TAC	-ATCACCGTCT	CTTCACAAT	-CACAAGACAC	AAGCAG(CTCCG- <mark>GTGAT</mark>	GAA <mark>A</mark> T	
	KF350033	GGGCTGCATGCCG	GAGCTG	CCTCACGACATCT	GGACGCTACA	GCAATATGCC	TAC	-ATCACGTGCG	CCTCTGAAG	-CGCAGGACAC	GACGAGC	AACG- <mark>GAAAC</mark>	GAGCT	
	KM402137 KM402138	GGGATGTATGCCZ	GAACTG	CCTCATCAAATCT CCCCACCACATCT	GGGAGCTCAC.	ACAAT ACGCC	TAT	- CTCACCATGC(- ATCACCTGCG)	CGTCGCAAG	-CGCAAGACAC	CGAAACO	IAATA-ACGTG	GAACT	
	NC_023673	Geectecatecce	GAGCIG	CCTCACGACATCT	GGACGCTACA	GCAATATGCC	TÂC	-ATCACGTGCG	CCTCTGAAG	-CGCAGGACAC	GACGAG(CAACG-GAAAC	GAGCT	
	NC015031	GGGATGCATGCC	IGAATTA	CCTCATGACATCT	GGGAACTGCC	GCAATACGCC	TAC	-ATCACCATGC	CGTCGCAGG	-CACAAGATAC	AGATAA	26 <mark>CT</mark> GA8	GAACG	
	NC016032 NC016647	AGGATGCATGCC	SCARCTC CARCTE	CCTCACGACGTCT CCGCACGACGTCT	GGARGETGER	ACAATACGCC CC SCT SCCCC	TAC	-ATCACGGTCG	CCCCTTATE	-CUCCARUACAUAU -Caraccarau	Galigga	reade- <mark>eceal</mark>	GAGCT	
	JF429834	GGGATGCATGCC	GAATTA	CCTCATGACATCT	GGGAACTGCC	GCAATACGCC	TAC	-ATCACCATGC	CGTCGCAGG	-CACAAGATAC	AGATAA(CGCTGAA	GAACG	
	JF429835	AGGATGCATGCC	GAACT G	CCTCACGA <mark>CG</mark> TCT	GGAAGCT <mark>GCA</mark>	ACAATACGCC	тас	-ATCACGGT <mark>CG</mark>	CCCCTTATG	-C <mark>G</mark> CAAGACAC	GACGGA	C <mark>G</mark> ACG- <mark>G</mark> CGA <mark>(</mark>	GAGCT	
	JF429835	AGGATGCATGCC	GAACTG	CCTCACGA <mark>CG</mark> TCT	GGAAGCT <mark>GCA</mark>	ACAATACGCC	TAC	-ATCACGGTCG	CCCCTTATG	-CGCAAGACAC	GACGGA(C <mark>G</mark> ACG- <mark>G</mark> CGAC	GAGCT	
	JF512472	AGGATGCATGCC	GAACTG	CCTCATGAAATCT CCTCATGAAATCT	GGGAACTTAC.	ACAATACGCC ACAATACGCC	TAC	- ATCATTGTTC	CATCACAAA CCTCCCAAA	-CACAAGACTC.	ATCTTCAACO	CAGTG-AAGAI	GAAGC	
	JF713714	AGGATGCATGCCG	GAGIIG GAATTG	CCACATGAAATAT CCACATGAAATAT	GGGAACTCCA.	ACAATACGCC ACAATACGCC	T AT	-CTCGTCGTAC	CTTCAGCCA	-CACAAGACIC	AAACGCCTCI	CAATG-AAAAT	GAAAA	
	JF713715	AGGATGCATGCCG	GA <mark>AT</mark> TG	CCTCATGACATCT	GGGAACTTCC	GCAGTACGCC	T AT	-ATCACGATGC	CTATECAAG	-CACAGGATAC	AGACAA(0 <mark>6</mark> 066A1	GAACG	PBoVG3
	JN621325	GGGATGCATGCCG	GGA <mark>A</mark> CT G	CCTCACGA <mark>CG</mark> TCT	GGACGCT <mark>GCA</mark>	ACA <mark>G</mark> TACGCC	ТАС	-ATCACAGTCG	CC <mark>AAT</mark> CATA	-CGCAGGACTC	G-ACAGA	CAACG- <mark>GCA</mark> AC	GAGCT	
	JN681175	GGGCTGTATGCC2	GAGCT A	CCTCAC <mark>AGC</mark> ATCT	GGAATCTGCA	ACAGTACOCC	TAC	-ATCACAGTCG	CATCTCAGG	-CGCAGGACAC	A-ACGGA	CAACG- <mark>CCA</mark> AC	GAGCT	
	JN831651 WC472562	AGGCTGCATGCCZ	GAGCTG	CCGCACGACATCT CCACATGAACTCT	GGACGCTGCA	GCAGTACGCC ACAATACGCC	ТАС Т ЪТ	-ATCACGTGCG	CCTCTGAGG CTTCACAGA	-CACAGGACAC -CACAAGACTC	G-ACGAGI	CAACG-GAAAC CAACG-CACAT	GAGCT	
	KF025378	GGGCTGCATGCCG	AAGCTA	CCGCACAGCCTAT	GGAATCTGCA	ACAGTACOCC	TAC	-ATCACE	c. I Chenvia	CISCINSVISC			Vinoc	
	KF025379	GGGATGCATGCCG	GAGCTG	CCTCA <mark>T</mark> GA <mark>A</mark> ATCT	GGGAACTCAC.	ACA <mark>G</mark> TACGCC	T AT	-CTCACGATAC	CGTCGCAAG	-CACA <mark>G</mark> GACAC	rGAAGA(CAATA- <mark>ATAC</mark> A	GAATT	
	KF025380	GGGCTGCATGCCG	GAGCTG	CCTCACGA <mark>C</mark> ATCT	GGACGCTGCA	GCAGTACGCC	TAC	-ATCACGTGCG	CCTCTGAAG	-CGCAGGACAC	A-ACGAG	CAACG- <mark>GAA</mark> AC	GAGCT	
	KF025381 WF025382	AGGATGTATGCCG	GAGTTG	CCTCATGAAATTT CCCCATGAAATTT	GGGGGGCTCAC	GCAATACGCC ACAATACCCC	TAT	-CTCACAATGC	CGTCACAAT CCTCACACA	-CACAGGACTC	rgaarc(CAACG-TCAAT	GAACT	
	KF025383	AGGATGCATGCCG	GAATTG	CCACATGACGITI	GGGCGCTACC.	ACAATACGCC	TAC	-ATCACCGTCA	GCTTACAAT	-CACAAGACTC	rgaaac	AAATA-CCAAC	GAGCT	
	KF025384	GGGCTGCATGCCG	GAGCT A	CCTCACAGCATCT	GGAATCTGCA	ACAGTACOCC	TAC	-ATCACGGTCG	CCTCTCAGA	-CGCAGGACAC	R-ACGGA	CAACG- <mark>CCA</mark> AC	GAACT	
	KF025385	GGGATGTATGCC	AGAACTG	CC <mark>ACATGACGTT</mark> T	GG <mark>GC</mark> GCTACC	acaata <mark>t</mark> gc <mark>a</mark>	TAC	- ATT ACGGT C A	GCT CACAAT	-CACAAGAC <mark>T</mark> C	гөааас	AAATA-CCAAC	GAACT	
	KF025385	AGGCTGCATGCCG	GAACTG	CCTCATGAAATTT	GGGAACTGAC	GCAATACGCC	TAT	-CTCACTATGC	CGGCGCAAG	-GACAAGACTC	FGATAC	CAATA-ATGTA	GAACT	
	KF025387	GGGATGCATGCC	SGAACTG SGAGCTG	CCICALGACATCI	GGGAGCTACC	ACAGTACOCC ACAGTACOCC	TAC	-ATTACCOLOGIC	CGGCACAGG CCTCTCAGA	-CACAAGACAG	G-GGAGA	PAARG-REGRE	GAGCT	
	KF025389	GGGATGCATGCCG	GAGCTG	CCTCACGAAGTAT	GGCAACTACC.	ACAATACGCC	TAT	-CTGACGGTCT	GCTCAGCAG	-CACAAGACTC	CTACGATCA	GAATG-CGAAT	GAAAA	
	KF025390	GGGATGCATGCCG	GGA <mark>A</mark> CT G	CCT C ACGA <mark>T</mark> AT CT	GGACACTOCA	ACA <mark>G</mark> TACOCC	TAC	-ATCACGGT <mark>CG</mark>	CC <mark>CCT</mark> CATA	-CACAAGACAC	GACGGA(C <mark>G</mark> ACG- <mark>G</mark> CGAC	GAGCT	
	KF025395	GGGCTGCATGCCG	GAGCTG	CCTCACGA <mark>C</mark> ATCT	GGACGCTGCA	GCAGTACGCC	TAC	-ATCACGTGCG	CCTCTGAGG	-CGCAGGACAC	G-ACGGA	CAACG- <mark>CCAAC</mark>	GAGCT	
	KIZUDI34	S'-ACTCTTTGCAGTO	TGACTC	CUTUACGACATUT ITC-3'	GGATGCTGCA.	acaaracecc	TAC	-arcaceer <mark>ce</mark>	CCGCTCATA 30	TATCCACTAT	GTCCGAG	TCTG-5	GAACT	-
				→						L	01000.00			
	1	PBoV G1-F							PB	oVG1-R				

3 Q	Consensus	GGGAACTAGATA	ATTTTGA	CETGECGAAAAG	GGACTITCAA	TCTGTCGCCAG	AGAAAATTGAAT	TACCTES	GACCATCACAG	AGAATTACT	ATTACTTT	AACGGTGTCTG	GA	
6	2 Sequences	3120	3130	3140	3120	3160	31140	3190	3130	3200	3210	3220	<u>.</u>	
н	Q223038	GGGCACAGACAGO	GTATTTGA	rgaaatgaaagc	TGAATTTCAA	ATTCGCTGCAG	GGATGGGAAAAT	T <mark>GAG</mark> T GG	CCTGATGCAAG	CAAAT GTT	GTTT AAATTT A	AAAAAAGCT AT A	GA 🖵	1
н	Q291308	CCCCACAGACAGC	CTATTON CTATTON	GAAATGAAAGC	TGAATTTCAA TCAATTTCAA	ATTEGETGEAG	GGATGGGAAAAT	reacted	CCIGALGCAAG	A AAT GTT	GTTT AAATTT &	BAAAAAGCT AT A	GA C X	
J. V	7025201	CCCC ACAGACAGE	GT ATTTGA	CAAATGAAAGC	IGAAITICAA ICAAITICAA	ATT COUT OURS ATT COUT OURS	GGAT GGGAAAAT		CCTGATGCAAG CCTGATGCAAG	A A AT OTT	GITTABATTIA CTTTABATTIA	SAAAAAGUTATA SSSSSSSCOTSTS	68	PROVCI
ĸ	J622366	GGGGACAGATAAC	GTGTTTGA	GAAATGAAAGC	TGAATTTCAA	TCAAGGTGCAA	AGAGGGAAAGGT	AGAATEG	GT AGAT GTT AG	AAATGCT	ATTTAGATTCA	AAAAAGCAATG	iga -	1 1 1 1 1 1
ĸ	J755665	AGGCACAGACAG	GTATTTGA	GAAATGAAAGC	TGAATTTCAA	ATTOGCTOCAG	GGATGGGAAAAT	TGAGTGG	CCTGATGCAAG	AAATGCT	GTTTAAATTTA	AAAAAAGCT AT A	GA	
K	R014255	AGGCACAGACAGO	GTATTTGA	GAAAT GAAAGC	T GAATTTCAA	ATTOGOTOCAG	GGATGGGAAAAT	T G <mark>AG</mark> T GG	CCTGATGCAAG	AAAT GYT	GTTTAAAATTTA	AAAAAAGCT AT A	GA	1
н	M053693	GGGCACAGATTAT	AT CTTT AC	GAGGGAATGAG	GGA <mark>T</mark> TT <mark>C</mark> CAA	AAACGCTGTAA	AGACAAT <mark>AA</mark> AT G	T <mark>G AG</mark> T G G	AAAGAT <mark>GT</mark> CAG	G <mark>AGAT CAT</mark>	GTTCGGCCTCA	AAAAAGGT CTT A	GA	
н	M053694	GGGCACAGATTAT	AT CTTT AC	GAGGGGATGAG	GGA <mark>T</mark> TT <mark>C</mark> CAA	AGACGCTGTA	AAGACAAT <mark>AA</mark> AT G	TG <mark>AG</mark> TGG	AAAGAT GT CAG	6 AAAT CAT	GTTCGGGTTT	AAAAAGAT CTTT	GA	
H	0291309	GGGCACTGATTAT	AT CTTT AC	GAGGGGATGAG	GGATTTCCAA	AAACGCTGTAA	AAGACAAT <mark>AA</mark> ATG	T G <mark>AA</mark> T G G	AAAGAT GT CAG	6 AAAT ACT	GTTCGGCCTCA	AAGAAGGTCCT2	s6A	
K	F025392	GGGCACT GATT AT	AT CTTT AC	GAGGGGATGAG	GGATTTTCAA	AAACGCTGTAA	AGACAAT AAAT G	T GAAT GG	AAAGATGTCAG	AAAT ACT	GTTCGGCCTG	AAAAAGGTCTTG	GA	
K	F025393	GGGCACTGATTAT	AT CTTT AC	CGAGGGGAATGAG	GGATTTCCAG	AAACGCTGTAG	AGACAATAAATG	TGAATGG	AAAGAT GT CAG	A A AT ACT	GTTCGGCCTG	AAAAAGGT CTT G	-6A - C X	
ĸ	T206155	GGGCACTGATTAT	ATCTTTAC	CAGGGGG AAAG	GGATTTTCAA GGATTTTCAA	AAACGTOCTAP	AGACAAI AAAI G	TGAATGG	ABAGAT GT CAG	A AAT ACT	GITCGGGITIZ	AAAAAGGT CTT 6	GA CA	
ĸ	F206157	GGGCACAGATTAT	ATCTTTAC	GAGGGGATGAG	GGATTTCCAA	AAACGCTGTAA	AGACAATAAATG	TGAATGG	AAAGATGTCAG	AAATACT	GTTCGGCCTCA	AGAAGGTCTTA	GA	PRoV CO
K	F205150	GGGCACT GATT AT	AT CTTT AC	GAGGGGCAGAG	GGATTTTCAA	AAACGCTGTAA	AGACAAT AAAT G	T <mark>G A A</mark> T G G	AAAGATGTCAG	GAAAT ACT	GTTCGGCCTCA	AGAAGGTCTTA	GA	100102
K	F205151	GGGAACT GATT AT	AT CTTT AC	GAGGGGCAGAG	GGA <mark>T</mark> TTTCAA	AAACGCTGTAA	AGACAAT <mark>AA</mark> AT G	T G <mark>AA</mark> T GG	AAAGAT GT CAG	6 AGAT CCT	GTTCGGCCTCA	AAA <mark>AA</mark> GGTCCT <mark>A</mark>	sGA	
K	F205153	GEGCACTGATTAT	ATCTTTAC	CEAGEGEATEAG	GGA <mark>T</mark> TTTCAA	AGACGTGGTAA	AAAACAAT AAAT G	T G <mark>AAT</mark> GG	AAAGAT GT CAG	GAAAT CCT	GTTCGGCTTC	AAGAAGGT ATT A	s6A	
K	F205155	GGGCACTGATTAT	GTCTTTAC	CGAGGGGATGAG	GGATTTCCAA	AAACGTGCTAA	AAGACAAT AAAT G	T <mark>GAA</mark> T GG	AGAGATGTCAG	CAA <mark>A</mark> TATT	GTTCGGGTTT	AAAAAGAT ACT A	GA	
K	F206167	GGGCACAGATTAT	GTCTTTAC	CGAGGGGATGAG	GAATTTTCAA	AAACGTGCTAA	AGACAATAAATG	TGAATGG	AGAGATETCAG	CAAATATT	GTTCGGGTTT	AGAAAAT ACT A	6A	
K V	W 402120	CCCC AC ACACT AT	ALALLIAU ATCTTTAC	CACCCCATCAC	SCATTTTCAA	ANACOULOIAN	ACALAALAALU		AAAGAIGICAG	CAANIACI	ATTCGGCCIC2	AGAAGGICIIA AAAAAGTCTTT	68	r
ท	IC 024453	GGGCACAGATTAT	ATCTTTAC	LONGOGGANTGAG	GGATTTCCAA	AAACGCTGTAA	AGACAATAAATG	TGAGTGG	AAAGATGTCAG	CAGATCAT	GTTCGGCCTCA	LAAAAGGT (TT A	GA	
ĸ	F205158	AGGAACTAGATGO	ATTTTTGA	GTGGGTAAAAA	AGAATTCCAA	TCAGTTGCCAG	ACAAAATTGCAT	TACATGG	GACCAACGTAG	AGAGTT ACT	TTTT ACGT AT A	AACGGTGCCTG	GA -	1
K	F206159	GGGAACTAGATA	ATTTTTGA	CAGGGCAAAGAA	AGACTTTCAA	TCAGTCGCCCG	AAAAATTGTAC	TA <mark>CG</mark> TGG	GACCA <mark>GCGCC</mark> G	AGAGTT ACT	ATT CACTT AT &	AAACGCTGTGTGTC	GA	ľ.
K	F205152	AGGAACT AGAT GO	ATTTTTGA	FGTGG <mark>GT</mark> AAAA <mark>A</mark>	AGAATTT CT A	TC <mark>A</mark> GTTGCCAG	ga <mark>c</mark> aaaatt g <mark>t</mark> at'	T Å <mark>C A</mark> T GG	GACCA <mark>AT GT</mark> AG	AG AGTT ACT	TTTT AC AT AT &	A <mark>g</mark> acgetg <mark>c</mark> tg	-GA	
K	F205154	GGGAACT AGGT AG	ATTTTTGA	CGTGGCGAAAAG	GGACTTTCAG	GCTGTCGCCCG	GGGAAATTGCAT	TACCIGG	GACCAACATAG	ACAATT ACT	ATTTAC GT AT &	AACG <mark>A</mark> TG <mark>CT</mark> TG	-GA	
K	F205155	AGGAACTCAGTGO	ATTTTTGA	GTGGGCAAAAG	AGAATTTCAA	TCTGTTGCCAG	ACAAAATT GT AT	T ACATGG	GACCA <mark>ATGCC</mark> G	AGAGTT ACT	ATTCACTTATA	AACGGTGTTTG	-GA	
K	1205155	AGGAACTAGATGO	ATTTTTGA	Forgoeraaaaa ateereaaaa	AGAATTCCAA	TCAGTIGCCAG	ACAAAATTGTAT	r ACATGG	GACCA <mark>ATGT</mark> AG	ACAGCTACT	TITTACGTAT &	SAACGGTG <mark>C</mark> CTG	-68 103	
K	M402137	GGGAACTAGATAC	ATTTTTGA	LAIGGCAAAAAA	AGACTITICAG	TCLOICOCCAP	AAATAATTGTAT	тасство	GACCAGCACCO GACCAGCACCO	ACAGTT ACT	ATTCACTTATA	AACGGTGTCTC AAACGGTGTCTC	IGA	
ĸ	M402138	GGGAACTAGATA	ATTTTTGA	CATGGCGAAGAA	AGACTTTCAG	TCTGTCGCCA	ACAAAATTGTGT	TACCTGG	GACCAGCACCG	A AATTACT	ATTTACTTTA	AACGGTGTCTG	GA	
N	IC_023673	GGGAACT AGAT AG	ATTTTTGA	C <mark>A</mark> TGGCGAAAA <mark>A</mark>	AGACTTT CAG	TCTGTCGCCA	ACAAAATT G <mark>T G</mark> T	CACCTGG	GACCA <mark>G</mark> CAC <mark>C</mark> G	AG AATT ACT	ATTT ACTTTT A	AAACGGTGTCTG	-GA	
N	015031	GGGAACT AG <mark>G</mark> T AG	ATTTTTGA	CGTGGCGAAAAG	GGACTTTCAG	GCTGTCGCCCG	GGGAAATTGCAT	TACCTGG	GACCAACATAG	AGAATT ACT	ATTTAC GT AT A	AAACG <mark>A</mark> TG <mark>CT</mark> TG	-GA	
N	016032	GGGAACTAGGTAG	ATTTTTGA	CGTGGCGAAAAG	GGACTTTCAG	GCTGTCGCCCG	GGGAAATTGCAT	TACCTOG	GACCAACATAG	AGAATTACT	ATTTACTTAT &	AACGATGTCTG	GA	
л Л	1010047	CCCAACT ACAT O	ATTTTCA	CTCCCCAAAAC	CCACTTTCAC	CCTCTCCCCCC	CCCALANTICCAL	TACCIGG	CACCAACATAC	AGA STTACT	ATTTACCIATA	NAACGAIGICIG	-68	
J	F429835	GGGAACT AGGT AC	ATTTTTGA	CGTGGCGAAAAG	GGACTTTCAG	GCTGTCGCCCG	GGGAAATT GCAT	TACCTES	GACCAACATAG	ACAATT ACT	ATTTACTTAT	AACGATGTCTG	GA	
J	F429835	GGGAACT AG <mark>G</mark> T AG	ATTTTTGA	CGTGGCGAAAAG	GGACTTTCAG	GCTGTCGCCCG	GGGAAATTGCAT	TACCTGG	GACCAACATAG	AGAATT ACT	ATTTACTT AT &	AACGATGTCTG	GA	
J	F512472	GGGAACT AG <mark>G</mark> T AG	ATTTTTGA	CGTGGCGAAAAG	GGACTTTCA <mark>T</mark>	GCTGTCGCCCG	GGGAAATT GCAT	T ACCT GG	GACCA <mark>A</mark> CA <mark>T</mark> AG	ac aatt act	'ATTT AC <mark>AT A</mark> T A	AAACG <mark>A</mark> TGTCTG	6A	
J	F512473	GGGAACT AGAT AG	ATTTTTGA	CATGGCAAAGAA	AGACTTTCAA	TCAGTOGCOAG	a <mark>aat</mark> aatt g <mark>t</mark> at'	TACCTGG	GACCAGCACCG	AGA <mark>G</mark> TT ACT	ATTCACTTAT	AAACGGTGTCTG	-GA	
J	F713714	GGGAACTAGGTAG	ATTTTTGA	CGTGGCGAAAAG	GGACTTTCAG	GCTGTCGCCCG	GGGAAATTGCAT	TACCIGG	GACCAACATAG	AGAGTT ACT	ATTTACTTAT &	NAACGATGTATG	GA	I PB₀V G3
J	N621325	GGGAACTAGATA	ATTTTTGA	FGTGGCAAAGAA	AGACTTTCAA	TCAGTOGCOAG	AAATAATTGTAT	тасство	GACCAGCACCG	AGAGCTATT	ATTOTOTATA	AACGGTGTCTC	iga	
J	N681175	GGGAACTAGATAG	ATTTTTGA	CATGGCGAAAAA	AGACTTTCAG	TCTGTCGCCA	ACAAAATTGTGT	TACCTGG	GACCAGCACCG	AAATTACT	ATTTACTTTA	AACGGTGTCTG	GA	
J.	1831551	GGGAACTAGATG	ATTTTTGA	F GT G G <mark>GT</mark> A A A A <mark>A</mark>	<mark>AGAATTC</mark> CAA	T CT GT <mark>T</mark> GC C AG	ACAAAATTGCAT	гасстве	GAT CAAT GT AG	GA <mark>G</mark> TT ACT	TTTTAC GT AT &	AAACG <mark>A</mark> TGTCTG	GA	
K	C473563	GGGAACT AGGT AG	ATTTTTGA	CGTGGCGAAAAG	GGACTTTCA <mark>G</mark>	GCT GT C G C C C	GGGAAATTGCAT	T ACCT GG	GACCA <mark>A</mark> CA <mark>T</mark> AG	AG AATT ACT	ATTT ACTT <mark>A</mark> T A	AAACG <mark>A</mark> TGTCTG	6A	
K	F025378	GGGAACT CGATAC	ATTTTTGA	CCTGGC <mark>A</mark> AAAAA	AGACTTTCAG	TCTGTCGCCA	ACAAAATT GT AT	CACCTGG	GACCA <mark>G</mark> CAC <mark>C</mark> G	AGA <mark>G</mark> TT ACT	ATTT ACTTTT &	AAACGGTGTCTG	-GA	
K	F025379	GGGAACTAGATAG	ATTTTTGA	CATGGCAAAAAA	AGACTTTCAA	TCAGTCGCCAG	AAATAATTGTAT	TACCTOG	GACCAGCACCG	AGAGTT ACT	ATTCACTTAT	AACGGTGTCTG	GA	
ĸ	F025381	GGGAACTAGGTAC	ATTTTTGA	LAI GGCGAAAAAG	GGACTTTCAG	GCTGCAGCCAR	GGGAAATTGCAT	тасство	GACCAGCACCO GACCAACATAG	ACAATTACT	ATTTACITIC	AACGATGCTC	iga	
ĸ	F025382	AGGAACTCAATAC	ATTTTTGA	CATGGCAAAGAA	AGACTTTCAA	TCAGTCGCCA	AAACAATTGTAT	гасстве	GACCAGCACCG	ACAGTT ACT	ATTCACTTATA	AACGGTGTCTG	GA	
K	F025383	GGGAACT AGGT AG	ATTTTTGA	CGTGGCGAAAAG	GGACTTTCA <mark>G</mark>	GCT GT C G C C G	RGGAAATTGCAT	гасстве	GACCA <mark>A</mark> CATAG	AGAATT ACT	ATTTAC AT AT A	AAACG <mark>A</mark> TG <mark>CT</mark> TG	6A	
K	F025384	GGGAACTCGATA	ATTTTTGA	C <mark>AT</mark> GGCGAA <mark>G</mark> AA	AGACTTTCAG	TCTGTCGCCA	AAC AAAAATT G <mark>T G</mark> T	CACCTGG	GACCA <mark>G</mark> CAC <mark>C</mark> G	AG AATT ACT	ATTT ACTTTT A	AAACGGTGTCTG	6A	
K	F025385	GGGAACT AG <mark>G</mark> T AG	ATTTTTGA	CGTGGCGAAAAG	GGACTTTCAG	GCTGTCGCCCG	GGGAAATTGCAT	TACCTGG	GACCAACATAG	AGAATT ACT	ATTTAC GT AT &	AAACG <mark>A</mark> TG <mark>CT</mark> TG	-GA	
K	F025385	GGGAACTCAATTC	ATTTTTGA	FGTGGCAAAGAA	AGACTTTCAA	TCAGTCGCCAG	AAATAATTGTAT	TACCTGG	GACCAGCACCG	AGA <mark>G</mark> TT ACT	ATTCTCTTATA	AACGGTGTCTG	-GA I	
K	TUZ3387	GGGAACTAGGTAG	ATTTTTGA	CFTGGCGGAAAAG	beautituae	GCTGTCGCCC6	HUGHAAATTUCAT	r actirige r actirige	GACCAACATAG	A ATTACT	ATTTAUGTATA	NAACGATGTCT6	-68 .c.s	
ĸ	F025389	GGGAACT AGAT GO	ATTTTTGA	F GT G G GT AAAAA	AGAATTCCAA	TCTGTTGCCAG	ACAAAATTGCAT	TACCTGG	GATCAATGTAG	CAGTT ACT	TTTTACGTAT	AACGATGCCTG	GA	
K	F025390	AGGAACTAGATG	ATTTTTGA	GT G G GT A A A A A	AGAATTYCAA	TCAGTT GCCAG	ACAAAATT G <mark>T</mark> AT	TAC <mark>A</mark> TGG	GACCANTETAG	AGA <mark>G</mark> TT ACT	TTTT ACGT AT &	AACGGTG <mark>C</mark> CTG	GA	
K	F025395	GGGAACTAGATA	ATTTTTGA	C <mark>AT</mark> GGCGAA <mark>G</mark> AA	AGACTTTCAG	TCTGTCGCCA	ACAAAATT G <mark>T G</mark> T	гасстве	GACCA <mark>G</mark> CAC <mark>C</mark> G	ac aatt act	ATTTACTTTA	AACGGTGTCTG	6A	
K	F205154	GGGAACT AGAT G	ATTTTTGA	r gt g g <mark>gt</mark> aaaa <mark>a</mark>	AGAATTCCAA	TCTGTTGCCAG	GA <mark>C</mark> AAAATTG <mark>C</mark> AT	гасстве	GA <mark>T CAACGT</mark> AG	AG <mark>AG</mark> TT ACT	TTTT AC <mark>GT A</mark> T &	AAACG <mark>A</mark> TG <mark>C</mark> CTG	iga —	1
	5'-	GGGCACTGATTA	TATCTTTA	-3			3	- TTACC	TTCTACAGTC	CC-5'				
	_			► /				•		_				
	PB	oV G2-F					Р	BoV G2-R						
				/										

Consensus	ACTCTTTGCAGTCTGAC	TCTTCTGTGGGC	GGTAACTTTGCG	SCETECCETCTT	TCATTTCAAAAAAC	AAATTGCGCCTG	GCGCTCAACGAG	CTA	AAGACACGGGGGAAC	
62 Sequences	3510 3520	3530	3540	3650 3	3660 3670	3280	3690	3700	3710 3720	
HQ223038	GTGAACTCGAACTAG	TTGG-		AG	AAAGAT C	CAACT GTT CC			AAAACTCAAAGACAT	-
HQ291308	GTGAACTCGAACTAG	TTGG-		-AG	AAA <mark>GAT</mark> C	CAACTGTTCC			AAAACTCAAAGACAT	
JX854557	GTGAACTCGAACTAG	TTGG-		AG	AAAGAT C	CAACTGTTCC			AAAACTCAAAGACAT	DD-MC1
KF025391	GTGAACTCGAACTAG	TTGG-		AG	AAAGATC	AACTGTTCC			AAAACTCAAAGACAT	L DO A GI
KJDZZ3DD	GCGGTCTACAACTTG	TAU		-T6	AAAAAA				AAACAACAAAAAAAA	
ND014255	GTGAACTCGAACTAG-	TIGG		- NG	22262TC	AACTGTTCC				
HM052592	AAGATETEGTGTTEGAT	CGTTCTTTCGGC	GOGTOGATCOGA	AAAGGGCGTGTT	TEENTTANACEAE	CANCI GITCC	COTTONACEAG	ADDONTO	AAACAGAACACGGGGGGGGAC	_
HM052594	AAGATETEGAGTEEGAE	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TEGACTGANACGAG	GATEGCACEG	CGCTCGACGAG	CCTCGGGC	AAACAAAAACACGGGGGGGGAC	
H0291309	AAGATCTCGAGTCCGAT	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TEGACTCANACEAE	GATEGCACCGA	ACGETEGACGAG	CCTCGGGC.	AAACAGAACACGGGGGGGGAC	
KF025392	AAGATETEGAGTEEGAT	CETTOTTCGEC	GGGTGGATCGGA	AAAGGGCGTGTT	TEEACTCAAACEAE	GATEGCACEGA	CGCTCGACGAG	CCTCGGGA	AAACAGAACACGGGGGGGAC	
KF025393	AAGATCTCGAGTCCGAT	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TEGACTCAAACGAG	GATCGCACCGA	CGCTCGACGAG	CCTCGGGC.	AAACAGAACACGGGGGGGTC	
KF205155	AAGATCTCGAGTCCGAC	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TEGACTCAAACGAG	GATCGCACCG	CGCTCGACGAG	CCTCGGGC.	AAACAGAACACGGGGGGGGAC	
KF205155	AAGATCTCGAGTCCGAC	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TGGACTCAAGCGAG	GATCGCACCG	ACGCTCGACGAG	CCTCGGGA	AAACAA <mark>A</mark> ACACGGGGGG <mark>G</mark> AC	
KF205157	AAGATCTCGAGTCCGAC	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TEGACTEAAACEAE	CGATCGCACCGG	GCGCTC <mark>G</mark> ACGAG <mark>1</mark>	CCTCGGGA	AAACAAAAACACGGGGGGGGAC	PBoV G2
KF205150	AAGATCTCGAGTCCGAC	CGTT CTTT CGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TGGACTCAAGCGAG	GATCGCACCG	ACGCTCGACGAG	CCTCGGGC.	AAACAGAACACGGGGGGGGAC	
KF205151	AAGATCTCGAGTCCGAC	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TEGACTCAAACGAG	GATCGCACCGA	ACGCTCGACGAG	CCTCGGGC.	AAACAGAACACGGGGGGGGAC	
KF205153	AAGATCTCGAGTCCGAC	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TGGACTCAAACGAG	CGATCGCACCG	ACGCTCGACGAG	CCT CGGGC.	AAACA <mark>GA</mark> ACACGGGGGG <mark>G</mark> AC	
KF205155	AAGATCTCGAGTCCGAT	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGT GT GTT	TGGACTCAAACGAG	CGATCGCACCG	ACGCTCGACGAG	CCTCGGGC.	AAACA <mark>GA</mark> ACACGGGGGG <mark>G</mark> AC	
KF205157	AAGATCTCGAGTCCGAC	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TGGACTCAAACGAG	CGATCGCACCGA	ACGCTCGACGAG	CCTCGGGC.	AAACAGAACACGGGGGGGAC	
KJ755666	AAT AT CT CGAGT CCGAT	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TGGACTCAAACGAG	CGATCGCACCG	ACGCTCGACGAG	CCTCGGGC.	AAACAGAACACGGGGGGGAC	
KM402139	AAGATCTCGAGTCCGAC	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TGGACTCAAACGAG	CGATCGCACCGA	ACGCTCGACGAG	CCTCGGGC	AAACAGAACACGGGGGGGTC	
NC_024453	AAGATCTCGTGTTCGAT	CGTTCTTTCGGC	GGGTGGATCGGA	AAAGGGCGTGTT	TGGACTCAAACGAG	CGATCGCACCGA	ACGCTCGACGAG	CCTCGGGA	AAACAGAACACGGGGGGGGAC	
KF205158	ACT CTTT & C A & T C T & A C	TCTTCTGTGGGC	GGTAACTTTGCG	GCGTGCCGTCTT	TCATTT <mark>G</mark> AAAAAAC	AAATTGCGCCTG	GCGCTCAACGAG	CT A	AAGACACGGGGGGAAC	
KF205159	ACTCTTTGCAGTCTGAC	TCTTOTETEEEC	GGTAACTTTGCG	GCGTGCCGTCTT	TCATATCAAAAAAC	AAATTGCGCCTG	GCGCTCAACGAGO	CT A	AAGACACGGGGGAAC	
KF205152	ACTCTTTGCAGTCTGAC	TCTTOTETEEEC	GGTAACTTTGCG	GCGTGCCGTCTT	TCATAT AAAAAAAA	AAATTGCGCCTG	GCGCTCAACGAGO	CT A	AAGACACGGGGGAAC	7
KF205154	ACTCTTTGCAGTCTGAC	TCTTOTETEEEC	GGTAACTTTGCG	GCGTGCCGTCTT	TCATTTGAAAAAAC	AAATTGCGCCTG	GCGCTCAACGAGG	CTA	AAGACACGGGGGAAC	
KF205155	ACTCTTTGCAGTCTGAC	TCTTOTETEEEC	GGTAACTTTGCG	GETGCCGTCTT	TCATTTAAAAAAAC	AAATTGCGCCTG	CGCTCAACGAG	CTA	AAGACACGGGGGAAC	
kF205158	ACTCTTTGCAGTCTGAT	TUTTOTETEEEC	GGTAACTTTGCG	CGTGCCGTCTT	TCATTIGAAAAAAC	AAATTGCGCCTG	CGCTCAACGAG	CTA	AAGACACGGGGGAAC	
KF350033	ACTCTTTGCAGTCTGAC	TCTTOTETEEEC	GGTAACTTTGCG	CATGCCGTCTT	TCACTTGAAAAAAC	AAATTGCGCCTG	CGCTCAACGAG	CTA	AAGACACGGGGGAAC	
KM402137	ACTCTTTGCAGTCTGAC	TCTTOTETEEEC	GGTAACTTTGCG	CGTGCCGTCTT	TCATATAAAAAAAC	AAATTGCGCCTG	CGCTCAACGAG	CTA	AAGACACGGGGGAAC	
KM402138 NC 022572	ACT CTTT GC AGT CT GAC	TCTTOTTGGGC	GGT AACTTT GCG	AAGGCCGTCTT	TCATATCAAAAAGC	AAAT <mark>A</mark> GCGCC <mark>C</mark> G AAATTGCGCCCCG	CGCTCAACGAA	CGA	AAGACACGGGGGGAAC	
NC016023	ACTICITIOCAGICIGAC	TUTTUTOTOGOGU	GGTAACTITOUS	CALOCCOLCTL COMPONENTS	TLATTCASSAG	ANALIGUGUUTS AAATTGCCCCTC	COCICARCOAGU	CT &	AAGACACOGOGOAAC	
NC016032	ACTETTTGCAGTETGAG	TCTTOTCTCCCC	GCTAACTIIGCO	COLOCCALCII	TCALLGAGAGAGA	AAATTGCGCCT6	COCTCARCORO	CTA		
NC016647	ACTETTTCCACTETCAC	TETTOTOTOGOC	CCTAACTITCCC	SCOLOCCOLCLE	TC 1T 1T 1 1 1 1 1 1 1 1 CC	AAATTCCCCCTC	CCCTCAACCAAC	CT 3	11C1C1CCCCCC11C	
12420824	ACTOTTTCCACTORCAC	Terrecoc	CCTAACTITCCC	SCOLOCCOLCLI	TCATTTCALLAR	AAATTGCGCCTG	COCT CAACOAA	CT A	\\C\C\CCCCCC\\C	
12423034	ACTOTTTCCACTORCAC	Terrerecce	CCTAACTIICCO	SCOLOCCOLCLI	TCALLGARAGE	AAATTCCCCCTC	COCTCANCOAGO	CT Assess	>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>	
JF429835	ACTETTTGCAGTETGAG	TCTTOTCTCCCC	GCTAACTIIGCO	COLOCCALCII	TCALLGAGAGAGA	AAATTGCGCCT6	COCTCARCORO	CTA		
JF512472	ACTETTTGCAGTETGAC	TCTTOTOTOTOGGG	GGTAACTITGCG	SCOTOCCOTOT	Trattralassac	AAATTGCGCCTG	CCCTCAACCAAC	CT 1	% % CACACOGOGO % AC	
JE512472	ACTOTTTGCASTOTGAC	TCTTOTOTOGGG	GGTAACTTTGCC	SCOTOCCOTOT	TCATATAAAAAAAA	A A ATT GC GC CT C	CCCTC AACCGGG	CT 3	aacararccccaar	
JE713714	ACTICTTTGCAGTCTGAC	TCTTOTOTOTOGG	GGTAACTTTGCG	SCOTOCCOTOT	TCATTIGAAAAAAA	AAATTGCGCCTG	SCOCT CAMCOOC	CTA	AAGACACGGGGGAAC	
JE712715	ACTICTTTGCAGTCTGAC	TOTTOTOTOTOTO	GGTAACTTTGCG	SCOTOCCOTOT	TCATTICAAAAAAA	AAATTGCGCCTG	COCT CAACOAGO CCGCTCAACGAGO	CT 1	AAGACACGGGGGAAC	PBoV G3
JN621225	ACTETTTGCAGTETGAC	TCTTOTOTOTOGGG	GGTAACTTTGC	CETECCETCTT	TCATATAAAAAAA	AAATTGCGCCTG	COCT CAACOAGO CCCCTCAACCAGO	CT 1	% % CACACOGOGOGA % C	1 1001 00
JN681175	ACTICTTTGCAGTCTGAC	TCTTOTTTGGGC	GGTAACTTTGCC	SAAGGEEGTETT	TCATATCAAAAAAC	AAGT AGC GC CT C	GEGETERALGAA	CAA	AAGACACGGGGGAAC	
JN831651	ACTICTTTGCAGTCTGAC	TCTTOTETEEEC	GGTAACTTTGCG	SCETECCETCTT	TCATATAAAAAAGC	AAATTGCGCCTG	SCGCTCAACGAA	ста	AAGACACGGGGGAAC	
KC473563	ACTETTTGCAGTETGAC	TCTTOTOTOTOGOGC	GGTAACTTTGCG	SCETECCETCTT	TCATTTGAAAAAAAC	AAATTGCGCCTG	GEGETEAACGAG	ста	AAGACACGGGGGAAC	
KF025378	ACTETTTGCAGTETGAC	TCTTOTTTGGGC	GGTAACTTTGC	AAAGGCCGTCTT	TCATATCAAAAAGC	AATAGCGCCCC	FEGETEAACGAA	CGA	AAGACACGGGGGAAC	
KF025379	ACTETTTGCAGTETGAC	TCTTOTGTGGGG	GGTAACTTTGCG	GETGCCGTCTT	TCATTIGAAAAAAC	AAATTGCGCCTG	CGCTCAACGAG	ста	AAGACACGGGGGAAC	
KF025380	ACTOGCTGCAGTOTGAC	TCTTOTTTGGGC	GGTAACTTTGCG	AAGGCCGTCTT	TCATATCAAAAAGC	AAATAGCGCCCG	GCGCTCAACGAA	CGA	AAGACACGGGGGAAC	
KF025381	ACTETTTGCAGTETGAC	TCTTOTETEEEC	GGTAACTTTGCG	SCGTGCCGTCTT	TCATTTGAAAAAAC	AAATTGCGCCTG	GCGCTCAACGAG	ста	AAGACACGGGGGGAAC	
KF025382	ACTETTTGCAGTECGAT	TCTTOTGTGGGC	GGTAACTTTGCG	SCGTGCCGTCTT	TCATATAAAAAAGC	AAATTGCGCCTG	GCGCTCAACGAGO	ста	AAGACACGGGGGAAC	
KF025383	ACTETTTGCAGTETGAE	TCTTOTOTOGOC	GGTAACTTTGCG	SCETECCETCTT	TCATTIGAAAAAAC	AAATTGCGCCTG	GCGCTCAACGAGO	ста	AAGACACGGGGGAAC	
KF025384	ACTETTTGCAGTETGAE	TCTTOTTGGGC	GGTAACTTTGCG	GAAGGCCGTCTT	TCAT <mark>A</mark> TCAAAAAGC	AAAT <mark>A</mark> GCGCC <mark>C</mark> G	GCGCTCAACGA <mark>A</mark> C	CGA	AAGACACGGGGGAAC	
KF025385	ACTCTTTGCAGTCTGAC	тсттотегеес	GGTAACTTTGCG	SCETECCETCTT	TCATTT <mark>A</mark> AAAAAAC	AAATTGCGCCTG	GCGCTCAACGAGO	ста	AAGACACGGGGGAAC	
KF025385	ACTETTTGCAGTETGAC	TCTTOTGTGGGC	GGTAACTTTGCG	SCETECCETCTT	TCATTTGAAAAAAC	AAATTGCGCCTG	GCGCTCAACGAGO	ста	AAGACACGGGGGAAC	
KF025387	ACT CTTT GC AGT CT GAT	TCTTOTGTGGGC	GGTAACTTTGCG	SCGTGCCGTCTT	TCATTT <mark>A</mark> AAAAAAC	AAATTGCGCCTG	GCGCTCAACGAGO	ста	AAGACACGGGGGGAGC	
KF025388	ACTCTTTGCAGTCTGAC	TCTTOTTGGGC	GGT AAC CT AGC #	AAGGGCCGTCTT	TCAT <mark>A</mark> TCAAAAAGC	AAAT <mark>A</mark> GCGCCTG	GCGCTCAACGA <mark>A</mark> C	CGA	AAGAC <mark>G</mark> CGGGGGGAAC	
KF025389	ACT CTTT GC AGT CT GAC	TCTTOTETEEEC	GGTAACTTTGCG	GCGTGCCGTCTT	TCATTT G AAAAAAC	AAATTGCGCCTG	GCGCTCAACGAGO	ста	AAGACACGGGGGAAC	
KF025390	ACTETTTGCAGTETGAC	TCTTOTGTGGGC	GGTAACTTTGCG	SCETECCETCTT	TCATTT <mark>A</mark> AAAAAAC	AAATTGCGCCTG	GCGCTCAACGAGO	ста	AAGACACGGGGGAAC	
KF025395	ACTCTCTGCAGTCTGAC	TCTTOTTGGGC	GGTAACTTTGCG	GAAGGCCGTCTT	TCAT <mark>A</mark> TCAAAAAGC	AAAT <mark>A</mark> GCGCC <mark>C</mark> G	GCGCTCAACGA <mark>A</mark> C	CGA	AAGACACGGGGGAAC	
KF205154	ACTETTTGCAGTETGAC	TCTTOTGTGGGC	GGTAACTTTGCG	GCGTGCCGTCTT	TCATTT <mark>A</mark> AAAAAAC	AAATTGCGCCTG	GCGCTCAACGAGO	CTA	AAGACACGGGGGAAC	
5	- ACTCTTTGCAGTCTGAC	TCTTC -3					3'-	GATTTC	TGTGCCCCCTTG-	5'
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Terpertue (c)

Highlights

An EvaGreen®-based multiplex real-time PCR (EG-mPCR) with melting curve analysis was developed for simultaneous detection and grouping of PBoVs;

The assay is specific, sensitive and cost-effective;

This method could be an effective alternative for routine surveillance testing of multiple PBoVs in pigs.