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Development and validation of a multiplex conventional PCR assay for simultaneous detection and grouping of porcine bocaviruses

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Abstract

Porcine bocavirus (PBoV), a newly described porcine parvovirus, has received attention because it can be commonly identified in clinically affected pigs including pigs with post-weaning multisystemic wasting syndrome (PWMS) and pigs with diarrhea. In recent years, novel PBoVs have been identified and were classified into three genogroups, but the ability to detect and classify these novel PBoVs is not comprehensive to date. In this study, a multiplex conventional PCR assay for

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simultaneous detection and grouping of PBoVs was developed by screening combinations of mixed primer pairs followed by optimization of the PCR conditions. This method exclusively amplifies targeted fragments of 531 bp from the VP1 gene of PBoV G1, 291 bp from the NP1 gene of PBoV G2, and 384 bp from the NP1/VP1 gene of PBoV G3. The assay has a detection limit of 1.0×10^3 copies/μL for PBoV G1 4.5×10^3 for PBoV G2 and 3.8×10^3 for PBoV G3 based on testing mixed purified plasmid constructs containing the specific viral target fragments. The performance of the multiplex PCR assay was comparable to that of the single PCRs which used the same primer pairs. Using the newly established multiplex PCR assay, 227 field samples including faeces, serum and tissue samples from pigs were investigated. All three PBoV genogroups were detected in the clinical samples with a detection rate of 1.3%, 2.6% and 12.3%, respectively for PBoV G1, G2 and G3. Additionally, coinfections with two or more PBoV were detected in 1.7% of the samples investigated. These results indicate the multiplex PCR assay is specific, sensitive and rapid, and can be used for the detection and differentiation of single and multiple infections of the three PBoV genogroups in pigs.

Keywords: multiplex PCR; detection; grouping; porcine bocaviruses; prevalence rate

1. Introduction

Porcine bocavirus was first discovered in Swedish pigs with post-weaning

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multisystemic wasting syndrome (PMWS) in 2009 and was classified within the *Bocaparvovirus* genus (Blomström et al., 2009; Cadar et al., 2011; Cotmore et al., 2014). Bocavirus was discovered in both humans and animals and to date includes human bocavirus (HBoV) (Allander et al., 2005; Cashman and O'Shea, 2012), porcine bocavirus (PBoV) (Blomström et al., 2009), bovine parvovirus (BPV) (Chen et al., 1986), minute virus of canines (MVC) (Binn et al., 1970), gorilla bocavirus (GBoV) (Kapoor et al., 2010a) and California sea lion bocavirus (CslBoV) (Li et al., 2011b). Viruses in the *Bocaparvovirus* genus belong to the subfamily *Parvovirinae* of the *Parvoviridae* family, which is a group of divergent linear ssDNA viruses (Tijssen et al., 2011). Porcine bocavirus has a genome of ~5 kb and has three open reading frames that encode four proteins: NS1, NP1, VP1 and VP2 (Choi et al., 2014; Zeng et al., 2011; Zhang et al., 2015b). Parvoviruses were demonstrated to have nucleotide substitution rates that are as high as those of some RNA viruses (Duffy, Shackelton, and Holmes, 2008; Shackelton et al., 2005) and recent results suggest that the presently circulating PBoVs exhibit considerable genetic diversity within the same sample and between different pigs (Lau et al., 2011; Liu et al., 2014; Shan et al., 2011; Zhang et al., 2014). These findings may indicate that these viruses are in the process of adaptation and can undergo rapid evolution to generate new genotypes or species. Numerous PBoVs have been discovered to date. These PBoVs have been classified into three genogroups, named provisionally G1, G2 and G3, based on phylogenetic analysis of the NS1, NP1 and VP1/2 coding genes and genomes (Gunn et al., 2015;

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Jiang et al., 2014; Xiao, Halbur, and Opriessnig, 2013; Yang et al., 2012).

Members of the genus *Bocaparvovirus* are pathogens that have been associated with various disease manifestations, including respiratory and enteric diseases (Jiang et al., 2014). All four genotypes of HBoV have been identified in children with acute gastroenteritis, whereas only HBoV1 and HBoV2 were identified in respiratory tract samples (Xu et al., 2012a). More recent research strongly supported the hypothesis that HBoV can cause severe acute respiratory tract infection in children in the absence of viral and bacterial co-infections (Broccolo et al., 2015; Moesker et al., 2015). Reports suggest that some PBoVs may be associated with respiratory signs or diarrhea, although the pathogenicity of PBoVs has also not been recognized clearly mainly due to a lack of a suitable cell culture system or animal model (Blomström et al., 2009; Zhai et al., 2010). Therefore, detection and differentiation of PBoV is important to better understand the potential associations between PBoVs and related diseases.

Recently, emerging parvoviruses in pigs were discovered through the application of random amplification and large-scale sequencing techniques, followed by bioinformatic analysis of large numbers of the sequences of the resulting clones (Allander et al., 2001; Allander et al., 2005; Shan et al., 2011). With public availability of these viral sequences, simple and sensitive PCR methods using specific primers have been developed for PBoV (Kapoor et al., 2010b; Lau et al., 2011).

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However, these methods usually focused on G1 of PBoVs mainly due to availabilty of limited numbers of reference strains and a consensus PCR assay using one primer pair targeting a conserved region to detect all the PBoVs has not been reported yet mainly due to the high level of genetic diversity. In addition, conventional PCR technology to detect several PBoV genogroups individually is labor-intensive and expensive. These limitations can be overcome by using a multiplex conventional PCR assay, which incorporates multiple primers that amplify RNA or DNA from several viruses simultaneously in a single reaction (Elnifro et al., 2000).

In the present study, a multiplex conventional PCR assay was developed by combining three pairs of primers in one reaction. Furthermore, the reaction conditions were optimized for the rapid detection and differentiation of PBoV G1, G2, and G3 on the basis of amplicon size. To validate its application, we tested this assay for specificity and sensitivity on clinical samples and compared the results with those obtained by using single PCRs.

2. Materials and methods

2.1. Viruses and samples

The following viruses were used and stored in our laboratory: PBoV G1 strain MN307 (GenBank Accession number KF025391), PBoV G2 strain IA147 (GenBank Accession number KF025392) and PBoV G3 strain IA270 (GenBank Accession

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number KF025390). To test specificity of the assay, the following non-targeted viruses were utilized: A commercial porcine parvovirus (PPV) vaccine strain (Beijing Zhonghai Animal Health Science and Technology Co., Ltd, China, No. 0040401), commercial transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) vaccine strains (Harbin Weike Biotechnology development company, Harbin, China, No. 030718), a classical swine fever virus (CSFV) (Hangzhou strain), porcine circovirus type 2 (PCV2) (GenBank Accession Number GQ996404) and porcine reproductive and respiratory syndrome virus (PRRSV) (GenBank accession number DQ269472). All viruses were maintained in the authors' laboratory.

A total of 227 pig samples were utilized to test the multiplex PCR. The samples included 200 faecal samples from clinically normal pigs located in the Zhejiang province and collected during 2013. In addition, 22 serum samples from normal pigs and five lung samples from pigs suffering from respiratory tract symptoms or reproductive failure were collected in different pig farms in several provinces in China during 2013 to 2014. All samples were stored at -80°C until testing. The samples used in this study were collected in accordance with international standards for animal welfare.

2.2. Primers design

All genomic sequences of the PBoVs utilized in this study were obtained from the

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GenBank nucleotide sequence database from the National Center for Biotechnology Information (NCBI). Highly conserved regions within each PBoV genogroup genomes were identified by alignment of all available 62 partial and complete or nearly complete genome sequences with Clustal W (DNAStar Inc., Madison, WI, USA) (Fig S1). Primers corresponding to these conserved regions of the viral genomes were designed using Primer Premier 5.0 (Primer Biosoft International, Palo Alto, CA, USA). Three pairs of primers were designed to amplify PBoV G1, PBoV G2 or PBoV G3 and are outlined in Table 1. The specificity of the primers was confirmed against random nucleotide sequences obtained by a BLAST search in the GenBank database. All primers were obtained from a commercial source (Sangon Biotech. Co., Ltd, Shanghai, China).

2.3. Nucleic acid extraction

The samples were processed as described previously (Jiang et al., 2014). 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 min. Viral genomic DNA was extracted from frozen clinical samples using the AxyPrep^{TM} Body Fluid Viral DNA/RNA Miniprep Kit 50-prep (Axygen, Hangzhou, China) according to the manufacturer's instructions. The extracted DNA was stored at -80°C until use.

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2.4. Single PCR and plasmid template construction

The PCR reaction for PBoV G1, G2 and G3 was conducted in a 25 μL mixture including 2.5 μL 10× PCR buffer, 1.2 μL 2.5 mM of each dNTPs, 2.5 μL 25 mM $MgCl_2$, 0.5 μL of each 10 μM primer (Table 1), 1.5 U of Taq DNA polymerase (5 U/μL) (Sangon), 2 μL of the DNA and 16 μL distilled water. The amplifications were performed in a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: after initial denaturation at 95°C for 3 min, 35 cycles were 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 aliquots through 1.5% agarose gel in 1×TAE (40 mM Tris-aceate [pH 8.0], 1 mM EDTA). Each specific viral target fragment was cloned into the plasmid pMD18-T (TaKaRa), and then sequenced and confirmed the constructed recombinant plasmids which were used as standard templates for optimization of the following PCR assays.

2.5. Optimization of the multiplex PCR assay

Based on the established single PCRs, a series of experiments were performed to optimize the multiplex PCR protocol, including reagent concentration and PCR cycling parameters. The multiplex PCR was carried out in a 20 μL reaction mixture consisting of 2 μL of 10× PCR Buffer, 0.1 to 6 mM $MgCl_2$, 0.01 to 0.6 mM dNTP mix, 0.5 to 6 U Taq DNA polymerase, 2 μL of each plasmid DNA, and 0.05 to 0.6 mM

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each primer pair of three PBoV genogroups. The amplification was run in a Bio-Rad thermal cycler (Laboratories, Hercules, CA, USA) under the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 s; annealing at 55-60°C for 30 s and elongation at 72°C for 30 s, followed by a final extension at 72°C for 10 min. Amplicons were detected by electrophoresing 5 μL aliquots through 1.5% agarose gel in 1×TAE buffer. Negative controls using distilled water instead of template DNA were run with each test.

2.6 Assay specificity of the multiplex PCR

The specificity of the multiplex PCR assay was verified by examining DNA/cDNA from the positive and negative controls. The size-specific PCR products (531, 291 and 384 bp) obtained were analyzed by electrophoresing, and the sequence identity was checked against sequences deposited in the GenBank using a BLAST search at NCBI site.

2.7 Assay sensitivity of the single PCR and multiplex PCR

In order to test and compare the sensitivity of the multiplex PCR versus single PCR, each specific viral target DNA fragment was cloned into the plasmid pMD18-T and served as template using a 10-fold dilution series. The number of gene copies per μL in each dilution was calculated by using the formula: copies/μL= $(6.02 \times 10^{23}) \times$ Plasmid concentration (ng/μL) $\times 10^{-9}$/[(Plasmid length (bp)$\times$ 660].

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2.8. Detection of PBoV G1, G2 and G3 in clinical specimens by the single and multiplex PCR

A total of 227 clinical specimens from pig farms in China were tested for PBoV G1, G2 and G3 by each single PCR and also by the combined multiplex PCR assay. Each specific viral target fragment was cloned into the plasmid pMD18-T and sequenced by Shanghai Sangon Biotechnology Co., Ltd.

3. Results

3.1. Optimization of the multiplex PCR

After optimization, the multiplex PCR produced amplicons of 531 bp for PBoV G1, 291 bp for PBoV G2 and 384 bp for PBoV G3 with clear and similar fluorescence intensity. The optimal annealing temperature was 56°C. Other optimal conditions determined for the multiplex PCR were as follows: a $MgCl_2$ concentration of 2.5 mM, a primer concentration of 0.2 μM for PBoV G1, 0.1 μM for PBoV G2 and 0.2 μM for PBoV G3, dNTP concentrations of 0.2 mM, and 2.5 U Taq DNA polymerase.

3.2. Specific detection of PBoV G1, G2 and G3 in the multiplex PCR assay

The multiplex PCR assay was able to detect and differentiate PBoV G1, G2 and G3 independently. When different combinations of the three PBoV group viruses were used in the multiplex PCR, the corresponding amplicons were produced and

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confirmed by sequencing (Fig. 1). In contrast, no amplicons were produced with the negative controls, which included PPV, PCV2, PRRSV, CSFV, TGEV, PEDV and ddH_2O (Fig. 1). These results indicate that the multiplex PCR assay was specific for detection and differentiation of the three main PBoV genogroups.

3.3. Sensitivity of the single PCR and multiplex PCR assays

The sensitivity of the single PCR assays were 1.0×10^3 genomic copies/μL of PBoV G1, 4.5×10^3 copies/μL of PBoV G2 and 3.8×10^3 copies/μL for PBoV G3 (Fig. 2A-C). The multiplex PCR assay was also able to detect at least 1.0×10^3 genomic copies/μL of PBoV G1, 4.5×10^3 copies/μL of PBoV G2 and 3.8×10^3 copies/μL for PBoV G3 (Fig. 2D).

3.4. Application of the multiplex PCR assay for clinical samples

To validate the multiplex PCR for diagnosis of PBoVs, 227 clinical specimens were tested for the three PBoV groups using the single and multiplex PCR assay and DNA sequencing of PCR positive products (Table 2). Among all samples 31/227 (13.7%) was tested positive by the multiplex PCR assay, which was slightly lower than 32/227 (14.1%) positive samples by the single PCRs. The detection rates were 1.3% for PBoV G1, 2.6% for PBoV G2 and 12.3% for PBoV G3. In addition, among the 227 samples, co-infection by PBoV G1 and PBoV G3 was detected in 0.4% of the samples, coinfection of PBoV G2 and PBoV G3 was detected in 0.9% of all samples and, all

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three PBoV genogroups were detected in 0.4% of the samples. Moreover, a higher prevalence rate of PBoVs (15%, 30/200 samples) was detected in faecal samples compared with serum (4.5%, 1/22 samples) and lung samples (0%, 0/5).

4. Discussion

Over the past six years, with increased availability of improved methods and techniques, the research on PBoV has expanded rapidly. Nevertheless, some questions remain unsolved. Previous PBoV DNA prevalence data depended on primer design using sequences of limited reference strains published in NCBI at that time, which was not comprehensive and may have resulted in non-ideal primer choices and false negative samples (Cadar et al., 2011; Lau et al., 2011; Li et al., 2011a; Shan et al., 2011; Yang et al., 2012; Zhai et al., 2010). To elucidate the biological characteristics and even the possible cross-species transmission of PBoVs and human BoVs, detection and further study of diverse PBoVs was necessary. A multiplex PCR has the potential to save substantial time, effort and reagent cost (Liu et al., 2013). In addition, it reduces the sample amount required for the assay, which is particularly important when sample material is limited. In order to detect all PBoVs infecting pigs using a single tool, a multiplex TaqMan real-time PCR assay has been recently described (Jiang et al., 2014). However, probe-based assays are expensive and highly influenced by the potential presence of mutations within the probe-binding site that can prevent annealing of the probe and subsequent detection (Zheng et al., 2012). In this study, a

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multiplex PCR method based on PBoV sequences described up to 2016 was successfully developed to detect and differentiate PBoV G1, G2 and G3 in samples from pigs with single or multiple infections.

The development of a multiplex PCR method is a complex task mainly due to the fact that the presence of more than one primer pair in the same reaction mix may limit the sensitivity or specificity or cause preferential amplification of specific targets (Elnifro et al., 2000; Markoulatos, Siafakas, and Moncany, 2002). The sensitivity of the multiplex PCR is usually approximately 10 fold lower than that of a single PCR (Xu et al., 2012b). However, the established multiplex PCR assay in the current study was as sensitive as the single PCR with a detection limit of 1.0×10^3, 4.5×10^3, and 3.8×10^3 copies/μL for PBoV G1, G2, and G3, respectively, indicating minimal interference among primers. Furthermore, the sensitivity was similar to that of a single PCR (10^3 copies/μL) (Liu et al., 2013) and a probe-based multiplex real-time PCR which had a detection limit around 600 copies/L for PBoV G2 (Jiang et al., 2014). This may suggest an ideal primer design and proper reaction optimization of the multiplex assay developed in this study, thus avoiding the possible formation of primer dimers among different targets in one reaction. These overall obtained results indicate that the multiplex PCR assay established here was sensitive for detection of all PBoV genogroups.

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Clinical samples may contain PCR inhibitors, which may be carried over during nucleic acid extraction and which can result in a reduction of the sensitivity of an assay potentially leading to inaccurate quantitative results or even false negative results (Hoorfar et al., 2004; Nolan et al., 2006). To account for this some PCR assays, mainly real time PCRs, have been developed to monitor sample extraction and amplification inhibitors by using an internal positive control (Auburna et al., 2011; Pal et al., 2008; Xiao et al., 2009). However, identification of PCR inhibitors was very limited in these studies. For example, only 2 of 1265 (0.2%) nasopharyngeal samples showed evidence of inhibition of amplification of the internal control in a multiplex real time PCR assay (Auburna et al., 2011). On the other hand, addition of an internal control increases the difficulty to optimize a multiplex assay and could compromise the sensitivity and specificity. Internal positive controls were therefore not included in this study.

To guarantee the specificity of the multiplex PCR, specific primers were designed in the highly conserved regions for each PBoV genogroup which were divergent among the three genogroups (Fig S1). The specific primers produced distinct PCR products for each PBoV group, which can be visualized and easily differentiated by 1.5% agarose gel electrophoresis. In addition, no amplification occurred with non-target viruses or ddH_2O, indicating that the multiplex PCR assay was specific for detection of the three targeted PBoV genogroups. Moreover, to verify the results of the

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multiplex PCR protocol, all the size-specific PCR products obtained by analyzing the clinical specimens were cloned into the plasmid pMD18-T and sequenced. The result of the BLAST analysis for these sequences indicated the identities of these size-specific PCR products, further confirming the specificity of the multiplex assay.

The multiplex PCR as an alternative laboratory method to standard PCR was evaluated in the current study. Two hundred and twenty-seven field samples were tested for the presence of the three PBoV genogroups and the result of the multiplex PCR was compared to that obtained by a standard PCR method. The results were identical, except for one sample that was tested positive for PBoV G2 by the single PCR, but negative by the multiplex PCR.

Among pig field samples tested, PBoV displayed an overall prevalence rate of 14.1%. This is similar to previous studies which reported that 12.59% (50/397) (Cheng et al., 2010), 11.4% (46/403) (Zhang et al., 2015a) and 16.5% (55/333) (Lau et al., 2011) of Chinese pigs samples were positive. Among the genogroups, PBoV G1, G2 and G3 had been reported to have varying prevalence rates ranging from 1.5 to 88% for G1, 4.8 to 64.4% for G2 and 8.7 to 81.3% for G3 in pig herds from different countries including China (Jiang et al., 2014; Xiao, Halbur, and Opriessnig, 2013; Zhang et al., 2015a). In the current study, the detection rate of PBoV G1, G2 and G3 was 1.3%, 2.6% and 12.3%, respectively. The variations in prevalence rates for each PBoV

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genogroup may be due to the differences in age of the pigs sampled, the source of samples and the detection limit of these assays. Nonetheless, similar to a previous study conducted in the American pig population, PBoV G3 was also the most frequently detected group circulating in Chinese pig herds, and coinfections of PBoV G1/G2, PBoV G1/G3, PBoV G2/G3 or PBoV G1/G2/G3 have also been demonstrated in a significant proportion of field cases (Jiang et al., 2014). The data obtained in the present study further confirm that co-infections with two or three PBoV groups are common in Chinese pigs.

In summary, the multiplex PCR assay described here provides a simple and sensitive tool for rapid detection of single as well as mixed infections of PBoV G1, G2 and G3, which are common in many pig herds. Therefore, this method could be a good alternative for routine molecular diagnosis or extensive epidemiological surveillance of PBoV in diagnostic laboratories.

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Table 1. Primers information for PBoV detection

Primer	Primer sequence (5′→3′)	Expected product (bp)	Target gene	Position	Reference sequence
PBoV G1-F	AATACCCATACTCACAAAG	531	VP1	3991-4521	HQ291308
PBoV G1-R	GTGATTGATTCATTGCTG				
PBoV G2-F	TCCACTGCTTCGAGAACATC	291	NP1	2519-2809	HM053693
PBoV G2-R	TTCCCTGACATCTTTCCATT				
PBoV G3-F	GAAATGTTAGAAGCTGTTGA	384	NP1/VP1	3098-3481	NC_016031
PBoV G3-R	TACAGGTGACGTTTATTGC				

Table 2. Frequency of viruses alone or in combination in 200 faecal samples from clinically normal pigs, 22 serum samples from healthy pigs and 5 tissue samples from clinically sick pigs collected from July 2013 to June 2014

Method	Sample Type	Health status[a]	No. samples	G1 [no. positive (%)][b]	G2 [no. positive (%)][b]	G3 [no. positive (%)][b]	G1 + G2 [no. positive (%)]	G1 + G3 [no. positive (%)]	G2 + G3 [no. positive (%)]	G1+ G2+ G3 [no.positive (%)]	Total [no. positive (%)]
Multiplex PCR	Faeces	H	200	1(0.5)	2(1)	23(11.5)	0(0)	1(0.5)	2(1)	1(0.5)	30(15)
	Serum	H	22	0(0)	0(0)	1(4.5)	0(0)	0(0)	0(0)	0(0)	1(4.5)
	lung	D	5	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

	Total		227	1(0.4)	2(0.9)	24(10.6)	0(0)	1(0.4)	2(0.9)	1(0.4)	31(13.7)
Singleplex PCR	Faeces	H	200	3(1.5)	6(3)	27(13.5)					31(15.5)
	Serum	H	22	0(0)	0(0)	1(4.5)					1(4.5)
	Lung	D	5	0(0)	0(0)	0(0)					0(0)
	Total		227	3(1.3)	6(2.6)	28(12.3)					32(14.1)

[a] H represents healthy; D represents diseased.

[b] G1, G2 and G3 represent PBoV G1, PBoV G2 and PBoV G2, respectively.

Fig. 1. Specificity of the multiplex polymerase chain reaction (PCR) assay developed for the detection of PBoV G1, G2 and G3. Agarose gel showing simultaneous multiplex PCR amplification of different combinations of viral genogroupes with the three sets of primers.

Fig. 2. Sensitivity of the single PCR assays for each PBoV genogroup (A, B and C) and the multiplex PCR for simultaneous amplification of the three PBoV genogroups (D). M: DL2000 DNA Marker. Lanes 1-7 are 1: 10^6 copies/μL; 2: 10^5 copies/μL; 3: 10^4 copies/μL; 4: 10^3 copies/μL; 5: 10^2 copies/μL; 6: 10^1 copies/μL; and 7: 10^0 copies/μL.

Fig S1 Primer position of three PBoV groups demonstrated by alignment of partial and complete or nearly complete genome sequences available in GenBank.