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# Accepted Manuscript

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Please cite this article as: Zheng X, Liu G, Opriessnig T, Wang Z, Yang Z, Jiang Y, Rapid detection and grouping of porcine bocaviruses by an EvaGreen® based multiplex real-time PCR assay using melting curve analysis, *Molecular and Cellular Probes* (2016), doi: 10.1016/j.mcp.2016.05.001.
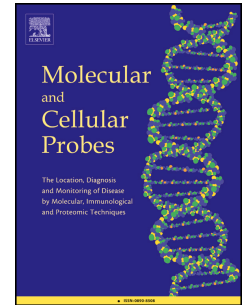
1  Rapid detection and grouping of porcine bocaviruses by an EvaGreen® based

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 ± 0.29°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

## 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

8 to the *Bocavirus* genus in the *Parvovirinae* subfamily of *Parvoviridae* family, which

9 is a group of divergent non-enveloped linear single stranded DNA viruses with a

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

12 encoding for the capsid proteins VP1 and VP2 [3]. Bocavirus, distinguished from

13 other parvoviruses by the presence of an additional third major ORF encoding for

14 NP1 of unknown function located in the middle of the viral genome, is known to

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

1    diarrhea, although the pathogenicity of PBoV has not yet been recognized clearly [8,

2    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

13    these studies. Thus, it is necessary to develop an effective and accurate approach to

14    detect PBoV but also to differentiate PBoV species for epidemiological surveillance

15    and to determine potential associations between PBoV and related diseases.

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

11 [15]. In order to detect all known PBoV species infecting pigs in clinical samples, a

12 multiplex real-time PCR assay has been recently described [5]. This method was

13 specific and sensitive for simultaneous detection and discrimination of all PBoVs that

14 were classified into three groups (PBoV G1, G2, and G3). However, TaqMan probes

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

19 In this study, we have developed an EvaGreen®-based multiplex real-time PCR

20 (EG-mPCR) assay followed by melting curve analysis for simultaneous detection of

21 all the different species of PBoV, allowing a rapid, sensitive and specific diagnosis of

22 PBoV infection including of identification of the viral species involved.

1

## 2. Materials and methods

3 2.1. Viruses and samples

4 PBoV G1 stain MN307 (KF025391), PBoV G2 stain IA147 (KF025392) and PBoV

5 G3 strain IA270 (KF025390) were maintained in the authors' laboratory. To test

6 specificity of the assay, the following non-targeted viruses were utilized: transmissible

7 gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) vaccine

8 strain (No. 030718, Harbin Weike Biotechnology development Company, Harbin,

9 China), classical swine fever virus (CSFV) (Hangzhou strain), porcine circovirus type

10 1 (PCV1, EF533941), porcine circovirus type 2 (PCV2, GQ996404) and porcine

11 reproductive and respiratory syndrome virus (PRRSV, DQ269472) were maintained in

12 the authors' laboratory.

13

14 A total of 227 pig samples collected from different pig farms in several provinces of

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

5    PBoV group genome were aligned with Clustal W (DNAStar Inc., Madison, WI, USA)

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

14   obtained by a BLAST search in GenBank databases from the National Center for

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

19   The samples were processed as described previously [5]. Briefly, tissue samples were

20   minced and diluted 1:10 (w/v) in Dulbecco's modified Eagle's medium, homogenized

21   and centrifuged at 1500 g for 10 min to obtain the supernatant. Faecal samples were

22   resuspended 1:10 (w/v) in PBS, vortexed for 30 s and centrifuged at 1500 g for 10

7

1    min. Viral genomic DNA was extracted from frozen clinical samples using the

2    AxyPrep^TM Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, Hangzhou, China)

3    according to the manufacturer's instructions. The extracted DNA was stored at

4    -80°Cμ 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

9    MgCl$_2$, 0.5 μL of each 10 μM primer (Table 1), 1.5 U of Taq DNA polymerase (5

10   U/μL) (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

14   extension at 72°C for 10 min. The amplicons were detected by electrophoresing 5 μL

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,

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 μM of the forward and reverse primers (Table 1), and 1 μL 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 μL 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) μM of each primer pair, and 2 μL 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**

13 3.1. Phylogenetic analysis and primer design of PBoV

14 Initially, available nucleotide sequences of PBoVs were aligned by the Clustal W

15 method and a phylogenetic tree of PBoVs was constructed based on partial and

16 complete or nearly complete genomes. Phylogenetic analysis revealed that these

17 PBoVs including 17 PBoV species clustered into three groups (PBoV G1, G2 and G3)

18 (Fig S1). Although the sequences displayed low similarity between groups, the PBoV

19 sequences within each group were found to be relatively conserved (Fig 1), and

20 specific primers targeting each PBoV group were successfully designed for

21 conventional and real-time PCRs (Table 1).

22

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

2    To determine the specificity of the assay, the three targeted and all non-targeted

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

5    no specific amplification was detected with the other non-targeted pig viruses

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 $81.3 \pm 0.34°C$ for PBoV G1, $78.2 \pm 0.37°C$ for

11   PBoV G2, and $85.0 \pm 0.29°C$ for PBoV G3 (Fig 2B). In addition, the specific

12   amplifications were also confirmed by electrophoresis with a 3% agarose gel (data not

13   shown). These results demonstrated that the EG-mPCR was specific for detection and

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 $1.0 \times 10^2$ copies/μL to $1.0 \times 10^7$ 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 $1.0 \times 10^6$ 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/μL 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

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

6    In addition, mixed infection of PBoV G1 and G2 was found in 3.1% of the samples,

7    PBoV G1 and G3 in 5.7%, PBoV G2 and G3 in 13.2%, and all three PBoV groups

8    were detected in 3.1% of the 227 samples when tested with the EG-mPCR assay. The

9    positive samples were confirmed by sequencing and all the sequences obtained

10    clustered in their respective groups by phylogenetic analysis. These results indicated

11    that the EG-mPCR could be applied for detection and differentiation of the three

12    PBoV groups in clinical samples and for epidemiological investigation.

13

14    **4. Discussion**

15    Since its discovery in 2009, PBoV has been detected globally. To date, eleven

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

1 and diseased pigs [14]. Similarly, human BoV1 (HBoV1) is known to be a respiratory

2 pathogen, while HBoV2-HBoV4 are likely putative agents causing gastroenteritis [23].

3 The findings in humans suggest that potential associations between PBoVs and related

4 diseases may exist. It is therefore important to develop an effective and reliable

5 method to demonstrate multiple PBoV species or genotypes with one single assay for

6 epidemiological surveillance and disease management of PBoV.

7

8 In the present study, all currently available partial and complete or nearly complete

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

14 diversity observed in PBoV, the sequences within each group are relatively conserved

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 $0.34°C$，$78.2 \pm 0.37°C$ and $85.0 \pm 0.29°C$, 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

1   peaks corresponding to primer dimer observed in this study, which was believed to

2   occur even under optimal conditions regardless of primer design and primer

3   complementarity [25], these small melt peaks could be easily discriminated from the

4   target amplicons because of a lower Tm value and signal strength. Furthermore,

5   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

8   could detect as few as 100 copies/μL for PBoV G1, 50 copies/μL for PBoV G2 and

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 $10^5$

12  copies/μL for PBoV [15], and even compared with TaqMan based real time PCR

13  which had detection limit of 600 copies/μL for PBoV2 [5]. Similar sensitivities for

14  EG-mPCR assays were also reported previously for simultaneous detection of six pig

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.

20  It is notable that with one PBoV group present at a fixed concentration of $1.0 \times 10^6$

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

5    PCRs which had a detection limit around $10^2$ copies/µL [27, 28]. In addition, the

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

13   in agreement with the EG-sPCR assay. Among the PBoV groups, PBoV G3 had the

14   highest overall prevalence of 41.9% and PBoV G1 had the lowest overall prevalence

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

8    Lau et al. first reported a Hong Kong pig infected with two subtypes of PBoV G3

9    (PBoV4-1 and PBoV4-2) [14]. Recent research indicated that co-infection with

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,

12   30]. In the current study, mixed infections of PBoV G1 and G2, PBoV G1 and G3,

13   PBoV G2 and G3 and PBoV G1, G2 and G3 were detected in 3.1%、5.7%、13.2% and

14   3.1% of 227 samples, respectively. Recombination, as an integral part of the evolution

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**

12

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.

17

18

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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/μL.

11

12

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/μL PBoV G2. (E)

15  Sensitivity for PBoV G2 with a background of $10^6$ copies/μL PBoV G3. (F) Sensitivity for PBoV

16  G3 with a background of $10^6$ copies/μL PBoV G2. (G) Sensitivity for three PBoV groups mixed in

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.

19

20

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.

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

| Primer | Primer sequence (5´ → 3´) | Expected product (bp) | Position | Reference sequence |
|---|---|---|---|---|
| Primers for conventional PCR and standard plasmid construction | | | | |
| 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 EvaGreen® single and multiplex real-time PCR 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 | | | |
| PBoV G3-F | ACTCTTTGCAGTCTGACTCTTC | 108 | 3328-3435 | NC_016031 |
| PBoV G3-R | GTTCCCCCGTGTCTTTAG | | | |

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

| PBoV | concentration （copies/μl） | Intra-assay | | Inter-assay | |
|------|------|------|------|------|------|
| | | 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 3. Detection of clinical samples by EG-mPCR and EG-sPCR

| Method | Sample Type | Health status | No. samples | PBoV G1 only [no. positive (%)] | PBoV G2 only [no. positive (%)] | PBoV G3 only [no. positive (%)] | PBoV G1 + PBoV G2 [no. positive (%)] | PBoV G1 + PBoV G3 [no. positive (%)] | PBoV G2 + PBoV G3 [no. positive (%)] | Three PBoV groups [no.positive (%)] | Total [no. positive (%)] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Multiplex real-time PCR | 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) |
| | Serum | Healthy | 22 | 0(0) | 2(9.1) | 5(22.7) | 0(0) | 0(0) | 9(40.9) | 2(9.1) | 18(81.8) |
| | 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 real-time PCR | 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) |
| | Serum | Healthy | 22 | 0 (0) | 0(0) | 3(13.6) | 0(0) | 0(0) | 13(59.1) | 2(9.1) | 18(81.8) |
| | 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) |

*

Fig 1

A

B

Fig 2

Fig 3

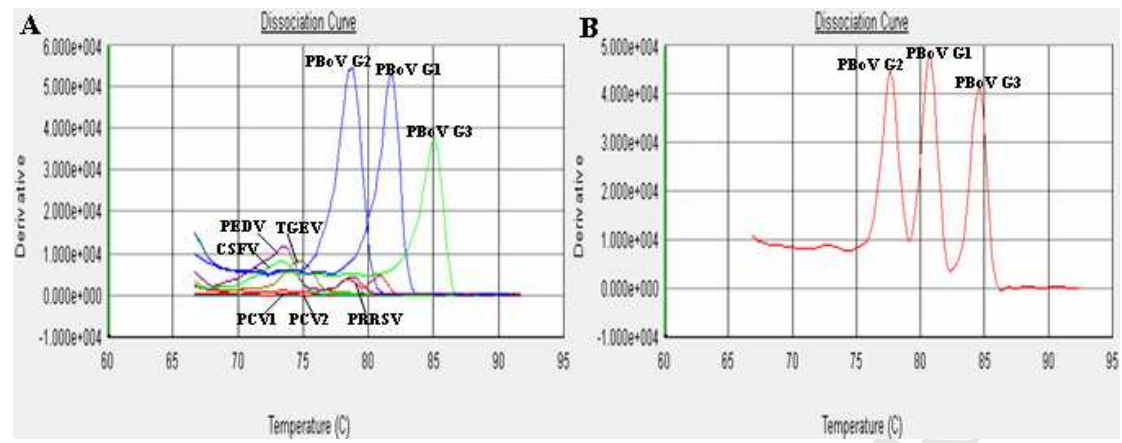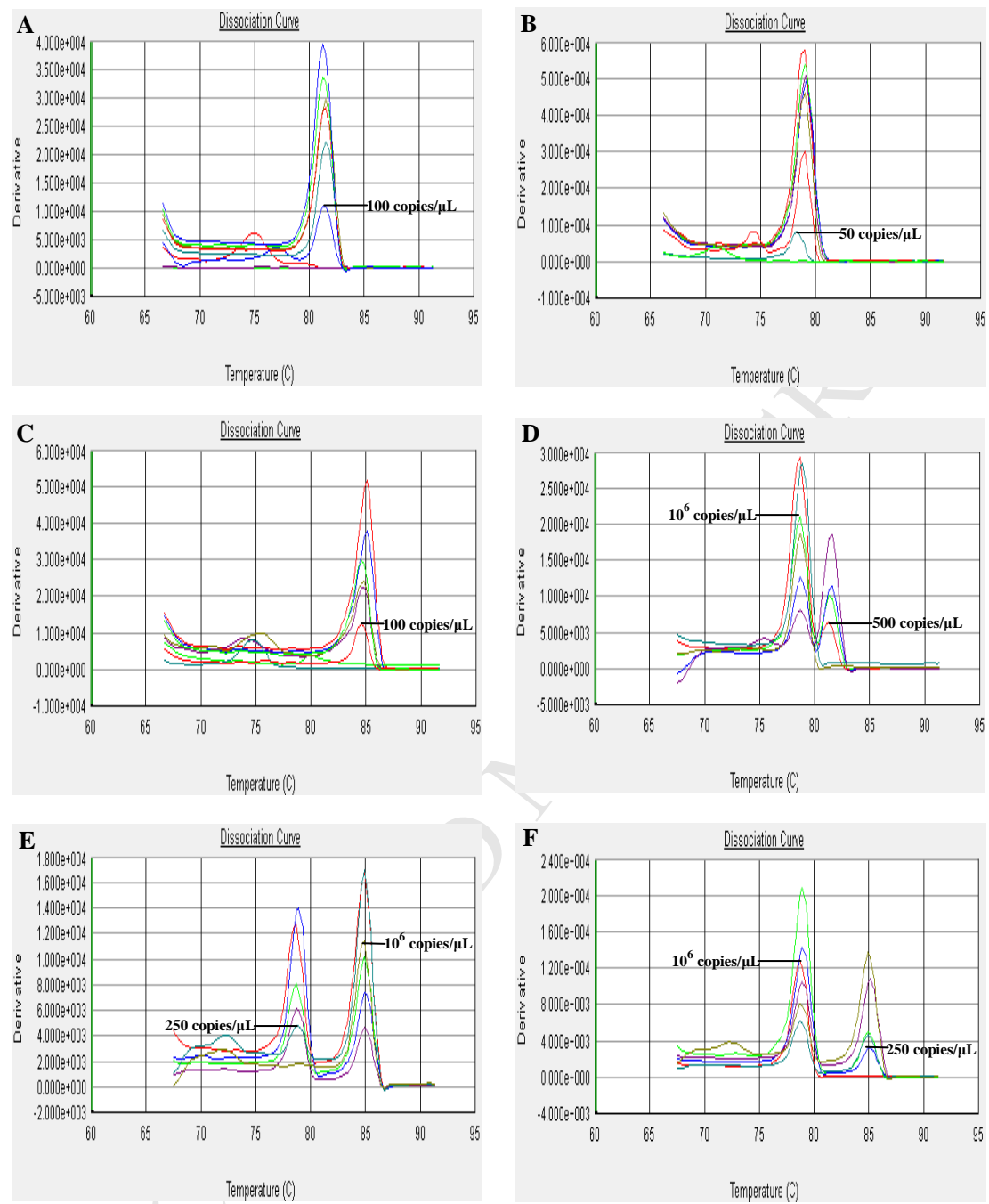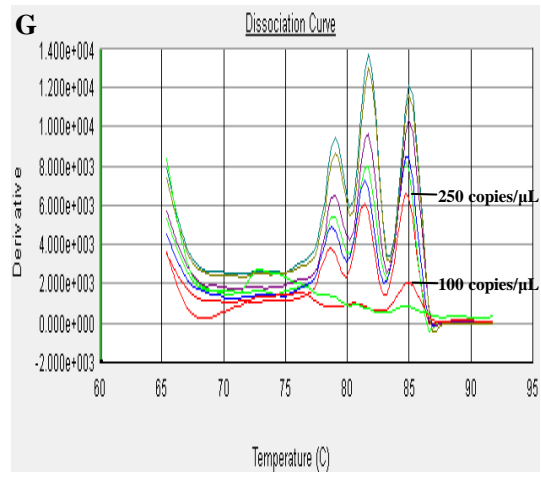G  Dissociation Curve

250 copies/μL

100 copies/μL

Temperature (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.