British Microbiology Research Journal
4(6): 607-615, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Concurrent Infection of Torque Teno Sus Virus and Porcine Circovirus Type 2 in a Clinical Case with Post-weaning Multisystemic Wasting Syndrome

Shao-Lun Zhai [1,2,3], Jin-Xue Long [2,4], Qin-Ling Chen[1], Man-Lin Luo[3], Wen-Kang Wei[1*] and Shi-Shan Yuan[2*]

[1]Institute of Animal Health, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China.
[2]Department of Swine Infectious Diseases, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, China.
[3]College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China.
[4]Department of R&D, Beijing CEVA-Huadu Biological Co., Ltd, Beijing 102600, China.

Authors' contributions

This work was carried out in collaboration between all authors. Authors SLZ and JXL designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors QLC and MLL managed the analyses of the study. Authors WKW and SSY revised the manuscript. Authors SLZ and JXL contributed equally to this study. All authors read and approved the final manuscript.

Short Communication

Received 9th August 2013
Accepted 26th November 2013
Published 20th February 2014

ABSTRACT

Aims: The goal of this study was to identify possible concurrent infection of torque teno sus virus (TTSuV) and porcine circovirus type 2 (PCV2) in a clinical case with post-weaning multisystemic wasting syndrome (PMWS) on certain farm of Shanghai, China.
Place and Duration of Study: Department of Swine Infectious Diseases, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, between June 2009 and June 2010 & Institute of Animal Health, Guangdong Academy of Agricultural Sciences, between September and November, 2013.
Methodology: Multiply-primed rolling-circle amplification (MPRCA), a useful molecular

**Corresponding author: Email: wwk188@tom.com; shishanyuan@hotmail.com;*

tool, was performed to amplify genome sequence of TTSuV and PCV2. For serum sample of SH0822 from a clinical case with PMWS, the products of MPRCA were digested using *Eco*R I, *Xba* I, *Sma* I, *Sac* I, respectively. Moreover, Clustal W program (DNASTAR software) and MEGA 5.1 software (neighbour-joining method) was used to analysis its nucleotide homology and genetic relationship.

Results: Restriction digestion analysis showed one TTSuV genome-size fragment was presented in 1.2 % agarose gel, moreover, another PCV2 genome-size fragment was also presented. Nucleotide sequencing and phylogenetic analysis results suggested that its complete genome were 2823-nucleotide size and 1767-nucleotide size and they were divided into species TTSuV1b and genotype PCV2b, respectively.

Conclusion: Concurrent infection of TTSuV and PCV2 in a clinical case with PMWS was identified using MPRCA combining with restriction endonuclease digestion, which indicated that MPRCA was an effective tool to attain simultaneous detection and genome amplification of TTSuV and PCV2.

Keywords: Torque teno sus virus; porcine circovirus type 2; multiply-primed rolling-circle amplification; complete genome; genetic relationship

1. INTRODUCTION

At the end of 20th century, torque teno virus (TTV) and porcine circovirus type 2 (PCV2) were firstly discovered in the serum of a Japanese patient and North American and European swine herds with wasting syndromes, respectively [1-3]. Since then, the two viruses were also identified in many kinds of animals, such as pets, rodents and economic animals [4-5]. Due to its circular genome, initially, both of them were divided into the same family/genus (Circoviridae family/Circovirus genus) in the previous study [6]. But now, the former belongs to *Anelloviridae* family, while the latter still belongs to Circoviridae family. For anelloviruses in pigs, its nomenclature was proposed and updated as Iotatorquevirus genus including the species of torque teno sus virus 1a and 1b (TTSuV1a and TTSuV1b) and Kappatorquevirus genus including the species of torque teno sus virus k2a and k2b (TTSuVk2a and TTSuVk2b) of *Anelloviridae* family [7]. Generally speaking, in aspect of genome, TTSuV (varied from 2.7 kb to 2.9 kb) [8-14] has much more information (such as G-C rich region and TATA box) than PCV2 (about 1.7 kb) [15-17].

Some DNA viruses, especially circoviruses, employ a rolling-circle mechanism for genome replication [18]. According to this principle, rolling-circle amplification assay (RCA) was first established in 1989 by using several DNA polymerases and different circular DNA templates [19]. Due to high-fidelity, strong strand-displacing capability, high processivity and proofreading activity, Phi29 DNA polymerase was chosen for RCA [20]. Unlike classic PCR, the primers of multiply-primed rolling-circle amplification (MPRCA) are random hexamers, which can amplify almost all of the circular DNAs in principle. Therefore, to some extent, MPRCA also can be used in viral metagenomics, especially circular viruses [8,21].

Under animal experiment conditions, PCV2 and TTSuV have been considered as causal pathogens of post-weaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS) [22-23]. Meanwhile, some epidemiological studies also showed that PCV2 and TTSuV had higher prevalence in disease-affected swine herds than in healthy swine herds clinically [17,24]. However, the results of latest studies suggested that TTSuV was frequently detected in healthy pigs or non-PMWS-affected pigs [25-27].

The goal of this study was to identify possible concurrent infection of TTSuV and PCV2 in a clinical case with PMWS on certain farm of China.

2. MATERIALS AND METHODS

In 2008, one serum sample (about 200 µl) from a clinical pig presenting PMWS, was submitted to Department of Swine Infectious Diseases, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences from one farm of Shanghai city. The sample was archived and stored at -80ºC until used. Viral nucleotides were extracted by Virus Genome Extract DNA kit according to the manufacturer's instructions (TIANGEN, Inc., Beijing, China). Subsequently, MPRCA was used to amplify complete genome of TTSuV and PCV2 according to the description of previous studies [8]. MPRCA products were digested with *EcoR* I, *Xba* I, *Sma* I and *Sac* I at 37ºC overnight, respectively. Digestion products were separated by 1.2% agarose gel (GENE TECH, Inc., Shanghai, China) and visualized by UV light exposure after ethidium bromide (EB) staining. The sizes of fragments corresponding to those of TTSuV (2.7 kb-2.9 kb) and PCV2 (1.7 kb) were purified (BioDev kit, Beijing, China), respectively. And then the objective amplicons were cloned into the plasmid vector PCR2.1 (Invitrogen Inc., Shanghai, China). Positive recombinant plasmids were sequenced using primers M13+ (5'-CAGGAAACAGCTATGACC-3') (for forward) and M13- (5'-TGTAAAACGACGGCCAGT-3') (for reverse) (Invitrogen, Inc., Shanghai, China). Moreover, some additional walking primers (not available) were also designed based on obtained sequences by Invitrogen Inc. for the sequencing of its complete genome. Obtained sequences (GenBank accession nos. GU450330 and GU450331) and reference sequences Table 1 were aligned by using the Clustal W program (DNASTAR software) to get its nucleotide homology. To further understand genetic relationship based on its complete genome, the phylogenetic tree was constructed by MEGA 5.1 software (neighbour-joining method).

3. RESULTS AND DISCUSSION

Through MPRCA and enzyme digestion, there were some different nucleotide fragment-size products presenting on 1.2% agarose gel. *EcoR* Ⅰ and *Sac* Ⅰ produced two bands (around 1700 bp and 2500-2700 bp). *Xba* Ⅰ yielded one strong band (about 1700 bp), meanwhile, *Sma* Ⅰ only yielded one 2700 bp band. Nucleotide fragments corresponding to the genome-size of *Anellovirus* (2500-2700 bp) and *Circovirus* (1700 bp) were purified, cloned and sequenced, respectively. The clone of about 2500-2700 bp sequence, named as PTTV1SH0822, was identified as TTSuV sequence (2535 bp, 2823 bp and 2775 bp respectively). Further analysis based on restriction maps showed there were two restriction sites for *Xba* Ⅰ and *Sac* Ⅰ enzymes during full-length genome of PTTV1SH0822 (2823 bp), so the fragments of 2535 bp and 2775 bp in length were not its complete genome. PTTV1SH0822 had a maximum nucleotide similarity of about 92.1 % with PTTV1b-VA strain (GenBank number: GU456384) and a minimum nucleotide similarity of about 70.4 % with TTV1_1914 strain (GenBank number: GU570200) in the same Iotatorquevirus genus. Meanwhile, the clone of about 1700 bp sequence, named as PCV2SH0822, was identified as PCV2 sequence (1, 767 bp) with a maximum nucleotide similarity of about 98.6 % with 48285 strain (GenBank number: AF055394) and a minimum nucleotide similarity of about 95.1% with DK1987PMWSfree strain (GenBank number: EU148504). Moreover, the phylogenetic tree also suggested that they were divided into species TTSuV1b and genotype PCV2b, respectively Fig. 1.

Table 1. Information of PCV2 and TTSuV full-length genomes used in this study

Type/species	Isolate	Source	GenBank No.	Genome Length (nt)	Reference
TTSuV1a	Sd-TTV31	Japan	AB076001	2878	[4]
TTSuV1a	TTSuV1_1914	Spain	GU570200	2913	[12]
TTSuV1a	TTV1_G21	Spain	GU570201	2910	[12]
TTSuV1a	FJ/China/2009/TTV1/9	China	JF937660	2872	[13]
TTSuV1a	GD/China/2009/TTV1/17	China	JF937662	2874	[13]
TTSuV1a	PTTV1a-VA	USA	GU456383	2878	[11]
TTSuV1b	1p	Brazil	AY823990	2872	[8]
TTSuV1b	swSTHY-TT27	Canada	GQ120664	2875	[9]
TTSuV1b	471819	Germany	GU188045	2863	[10]
TTSuV1b	PTTV1b-VA	USA	GU456384	2875	[11]
TTSuV1b	PTTV1SH0822	China	GU450331	2823	This study
TTSuVk2a	2p	Brazil	AY823991	2735	[8]
TTSuVk2a	472142	Germany	GU188046	2802	[10]
TTSuVk2a	PTTV2b-VA	USA	GU456385	2750	[11]
TTSuVk2a	PTTV2c-VA	USA	GU456386	2803	[11]
TTSuVk2a	TTV2_G31	Spain	GU570204	2744	[12]
TTSuVk2a	TTV2_G43	Spain	GU570206	2736	[12]
TTSuVk2a	TTV2_G64	Spain	GU570208	2745	[12]
TTSuVk2a	TTV2_GE1	Spain	GU570209	2742	[12]
TTSuVk2a	FJ/China/2010/TTV2/2	China	JF937656	2788	[14]
TTSuVk2a	GD/China/2009/TTV1/9	China	JF937659	2791	[14]
TTSuVk2b	38E05	New Zealand	JQ406844	2901	[7]
TTSuVk2b	38E19	New Zealand	JQ406845	2899	[7]
TTSuVk2b	38E23	New Zealand	JQ406846	2899	[7]
PCV2a	1010-Stoon	Canada	AF055392	1768	[2]
PCV2a	GX0841a	China	GQ359003	1768	[17]
PCV2b	48285	France	AF055394	1767	[2]
PCV2b	GX0841b	China	GQ359004	1767	[17]
PCV2b	PCV2SH0822	China	GU450330	1767	This study
PCV2c	DK1987PMWSfree	Denmark	EU148504	1767	[15]
PCV2d	TJ	China	AY181946	1767	[16]
PCV2d	BJ0901b	China	GU001710	1767	[17]
PCV2e	GX0601	China	EF524532	1768	[16]
PCV2e	BJ0901a	China	GU001709	1768	[17]

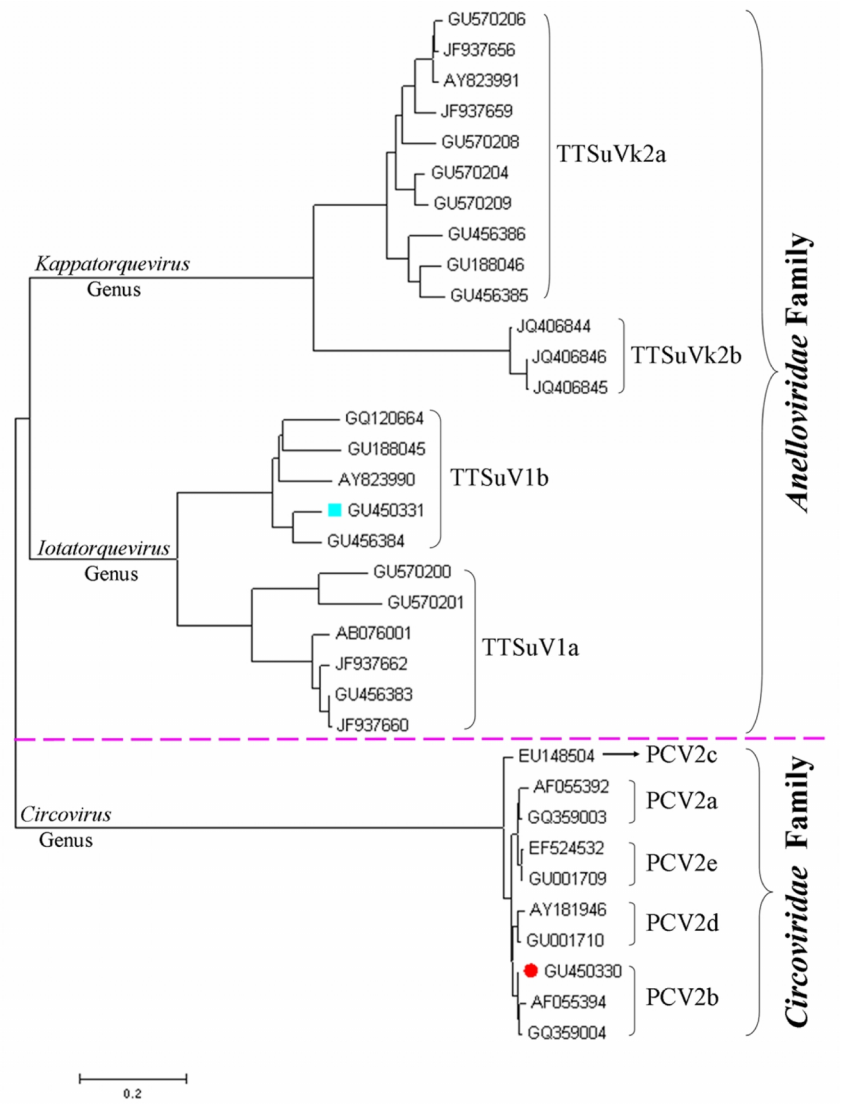

Fig. 1. Phylogenetic analysis of TTSuV and PCV2 strains based on its complete genome using the neighbor-joining method with MEGA 5.1 software. The reliability of the different phylogenetic groupings was evaluated by using the bootstrap test (1000 bootstrap replications). Note: PTTV1SH0822 clone (GenBank Number: GU450331) and PCV2SH0822 clone (GenBank Number: GU450330) were labelled by "■" and "●", respectively

RCA and its modified version, a useful molecular tool, was used to detect known viruses and discover new unknown viruses [7,8,28]. Moreover, it was also utilized to amplify complete genome of some circular viruses and made infectious clone of some virus easy, such as PCV2 [29]. However, using PCR method, complete genome of one circular virus (especially those viruses with long genome) needed be segmentally amplified for several times, such as TTSuV [11,13,14]. In this study, we used MPRCA to amplify genome of TTSuV and PCV2 in the sample of SH0822. Genarally speaking, if the detailed infection genotypes or species of

PCV2 and TTSuV were not considered, the detection results in this study were consistent with the results of classic PCR previously described [17,30]. In addition, 30 PMWS samples (tissue) and 30 clinical healthy samples were detected by MPRCA and PCR, the detection results suggested that both of them had the same detection effects. However, MPRCA could not effectively and intuitively identify the co-existence of multiple strains of PCV2 or TTSuV on agarose gel. In our previous study, using classic PCR, viral nucleotides (5' non-coding sequences) of PCV2b, TTSuV1a and TTSuVk2a were detected in the sample of SH0822 [17, 30]. Regretfully, in the present study, we only got complete genome of PCV2b and so-named "TTSuV1b" without TTSuVk2a. The possible reasons for this were as follows: (1) 5' non-coding genes were conserved for TTSuV1a and TTSuV1b, in fact, we found that there was only one nucleotide difference in 5' non-coding genes between TTV1-SH0822 (GenBank number: GQ358982) and PTTV1SH0822 (GenBank number: GU450331), so we should classify them according to certain reference genes, such as their ORF1 genes [7], otherwise the same gene would be divided into different species or genotypes according to different phylogenetic analysis; (2) Maybe, the concentration of TTSuV1b sequences digested by restriction enzyme was significantly higher than that of TTSuVk2a. When plasmid vector PCR2.1 was used to clone the sequences of TTSuV1b and TTSuVk2a, TTSuV1b sequences were easily inserted into vector PCR2.1. Therefore, the result was that five clones were sequenced and identified as TTSuV1b, while the full-length sequences of TTSuVk2a the strain were not obtained; (3) Initially, we assumed the possibility that PCR 2.1 vector being used for cloning was inefficient, and then plasmid vector pUC19 (Invitrogen Inc.) was also used, regretfully, the sequences of TTSuVk2a strains were still not obtained. Possibly, the TTSuVk2a strains lacked restriction sites of *EcoR* I, *Xba* I, *Sma* I and *Sac* I in its genome; (4) Isolations of several TTSuV full-length clones from the same enzyme digestion product should be a time-consuming process, it could take much time to obtain different clones. Therefore, it should be necessary for us to design species-specific or genotype-specific primers for amplifying the complete genomes of different circle DNA viruses [28].

4. CONCLUSION

In summary, we identified concurrent infection of TTSuV and PCV2 in a clinical case with PMWS using MPRCA combining with restriction endonuclease digestion, which indicated that MPRCA was an effective tool to attain simultaneous detection and genome amplification of TTSuV and PCV2. Meanwhile, complete genome of PTTV1SH0822 and PCV2SH0822 was cloned and analyzed, it was also helpful for us to understand genetic relationship of TTSuV and PCV2.

ACKNOWLEDGEMENTS

This study is part of the work supported by grants (No. 2012A020602075 and No. 2012224-26) funded by Guangdong Provincial Department of Science and Technology and Guangzhou Science and Technology and Information Bureau, respectively.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Nishizawa T, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem Biophys Res Commun. 1997;241(1):92–97.
2. Meehan BM, McNeilly F, Todd D, Kennedy S, Jewhurst VA, Ellis JA, Hassard LE, Clark EG, Haines DM, Allan GM. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J Gen Virol. 1998;79(Pt-9):2171–2179.
3. Morozov I, Sirinarumitr T, Sorden SD, Halbur PG, Morgan MK, Yoon KJ, Paul PS. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. J Clin Microbiol. 1998;36(9):2535–2541.
4. Okamoto H, Takahashi M, Nishizawa T, Tawara A, Fukai K, Muramatsu U, Naito Y, Yoshikawa A. Genomic characterization of TT viruses (TTVs) in pigs, cats and dogs and their relatedness with species-specific TTVs in primates and tupaias. J Gen Virol. 2002;83(Pt-6):1291–1297.
5. Zhai SL, Chen SN, Zhang JW, Wei ZZ, Long JX, Yuan SS, Wei WK, Chen QL, Xuan H, Wu DC. Dissection of the Possible Routes on Porcine Circoviruses Infecting Human. J Anim Vet Adv. 2012;11(9):1281–1286.
6. Miyata H, Tsunoda H, Kazi A, Yamada A, Khan MA, Murakami J, Kamahora T, Shiraki K, Hino S. Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. J Virol. 1999;73(5):3582–3586.
7. Cornelissen-Keijsers V, Jiménez-Melsió A, Sonnemans D, Cortey M, Segalés J, Van Den Born E, Kekarainen T. Discovery of a novel Torque teno sus virus species: genetic characterization, epidemiological assessment and disease association. J Gen Virol. 2012;93(Pt-12):2682–2691.
8. Niel C, Diniz-Mendes L, Devalle S. Rolling-circle amplification of Torque teno virus (TTV) complete genomes from human and swine sera and identification of a novel swine TTV genogroup. J Gen Virol. 2005;86(Pt-5):1343–1347.
9. Brassard J, Gagné MJ, Houde A, Poitras E, Ward P. Development of a real-time TaqMan PCR assay for the detection of porcine and bovine Torque teno virus. J Appl Microbiol. 2010;108(6):2191–2198.
10. Gallei A, Pesch S, Esking WS, Keller C, Ohlinger VF. Porcine Torque teno virus: determination of viral genomic loads by genogroup-specific multiplex rt-PCR, detection of frequent multiple infections with genogroups 1 or 2 and establishment of viral full-length sequences. Vet Microbiol. 2010;143(2-4):202–212.
11. Huang YW, Ni YY, Dryman BA, Meng XJ. Multiple infection of porcine Torque teno virus in a single pig and characterization of the full-length genomic sequences of four U.S. prototype PTTV strains: implication for genotyping of PTTV. Virology. 2010;396(2):289–297.
12. Cortey M, Macera L, Segalés J, Kekarainen T. Genetic variability and phylogeny of Torque teno sus virus 1 (TTSuV1) and 2 (TTSuV2) based on complete genomes. Vet Microbiol. 2011;148(2-4):125–131.

13. Wang MM, Zhou YJ, Zhang QZ, Hou JW, Yu H, Tong GZ. Complete genome sequences of highly divergent torque teno virus type I from swine herds. J Virol. 2012;86(21):11953
14. Wang MM, Zhou YJ, Zhang QZ, Hou JW, Yu H, Tong GZ. Complete genome sequences of highly divergent torque teno virus type II from swine herds. J Virol. 2012;86(22):12465.
15. Dupont K, Nielsen EO, Baekbo P, Larsen LE. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in genotypes with time. Vet Microbiol. 2008;128(1-2):56–64.
16. Wang F, Guo X, Ge X, Wang Z, Chen Y, Cha Z, Yang H. Genetic variation analysis of Chinese strains of porcine circovirus type 2. Virus Res. 2009;145(1):151–156
17. Zhai SL, Chen SN, Wei ZZ, Zhang JW, Huang L, Lin T, Yue C, Ran DL, Yuan SS, Wei WK, Long JX. Co-existence of multiple strains of porcine circovirus type 2 in the same pig from China. Virol J. 2011;8:517.
18. Cheung AK. Porcine circovirus: transcription and DNA replication. Virus Res. 2012;164(1-2):46–53.
19. Shavitt O, Livneh Z. Rolling-circle replication of UV-irradiated duplex DNA in the phi X174 replicative-form----single-strand replication system *In vitro*. J Bacteriol. 1989;171(6):3530-3538.
20. Blanco L, Bernad A, Lázaro JM, Martín G, Garmendia C, Salas M. Highly efficient DNA synthesis by the phage phi 29 DNA polymerase. Symmetrical mode of DNA replication. J Biol Chem. 1989;264(15):8935-8940.
21. Blomström AL. Viral metagenomics as an emerging and powerful tool in veterinary medicine. Vet Q. 2011;31(3):107**–**114.
22. Ellis JA, Allan GM, Krakowka S. Effect of coinfection with genogroup 1 porcine torque teno virus on porcine circovirus type 2-associated postweaning multisystemic wasting syndrome in gnotobiotic pigs. Am J Vet Res. 2008;69(12):1608–1614.
23. Krakowka S, Hartunian C, Hamberg A, Shoup D, Rings M, Zhang Y, Allan GM, Ellis JA. Evaluation of induction of porcine dermatitis and nephropathy syndrome in gnotobiotic pigs with negative results for porcine circovirus type 2. Am J Vet Res. 2008;69(12):1615–1622.
24. Kekarainen T, Sibila M, Segalés J. Prevalence of swine Torque teno virus in post-weaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs in Spain. J Gen Virol. 2006;87(Pt-4):833–837.
25. Li K, Wang LQ, Wu YY, Chao AJ, Lu QW, Wei ZY, Cui BA, Chen HY. Molecular detection and genomic characterization of Torque teno sus virus 1 and 2 from domestic pigs in central China. Virus Genes. 2013;46(3):479-486.
26. McMenamy MJ, McKillen J, McNair I, Duffy C, Blomström AL, Charreyre C, Welsh M, Allan G. Detection of a porcine boca-like virus in combination with porcine circovirus type 2 genotypes and Torque teno sus virus in pigs from postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected farms in archival samples from Great Britain. Vet Microbiol. 2013;164(3-4):293-298.

27. de Arruda Leme R, Ribeiro J, Alfieri AF, Alfieri AA. Simultaneous infection with distinct strains of torque teno sus virus (TTSuV) in healthy slaughter-age pigs. Vet Res Commun. 2013;37(2):183-186.
28. Macera L, Cortey M, Maggi F, Segalés J, Kekarainen T. A novel rolling circle amplification assay to detect members of the family Anelloviridae in pigs and humans. Virus Res. 2011;160(1-2):424–427.
29. Dezen D, Rijsewijk FA, Teixeira TF, Holz CL, Cibulski SP, Franco AC, Dellagostin OA, Roehe PM. Multiply-primed rolling-circle amplification (MPRCA) of PCV2 genomes: applications on detection, sequencing and virus isolation. Res Vet Sci. 2010;88(3):436–440.
30. Zhai SL, Long JX, Wei WK, Chen QL, Luo ML, Lv DH, Wu DC, Gao F, Yuan SS, Tong GZ, Wei ZZ. High prevalence of torque teno sus virus in China and genetic diversity of the 5' non-coding region. Arch Virol. 2013;158(7):1567-1573.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=432&id=8&aid=3769