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Short communication

An epidemiological investigation of porcine circovirus 3 infection in dogs in the Guangxi Province from 2015 to 2017, China

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ABSTRACT

Porcine circovirus type 3 (PCV3) is an emerging circovirus species associated with several diseases. The study aimed to investigate the frequency of porcine circovirus 3 (PCV3) and its coinfection with canine parvovirus type 2 (CPV-2) in dogs in the Guangxi province from 2015 to 2017, China, and to examine the genome diversity of PCV3. Using polymerase chain reaction (PCR) amplification and sequencing, 96 of 406 (23.6%) samples were positive for PCV3, 38 out of 406 (9.4%) samples were coinfecting with both PCV3 and CPV-2. The CPV-positive rate was significantly higher in the PCV3-positive samples than in the non-PCV3 samples, and the difference was extremely significant ($P < 0.01$). The complete genome ($n=4$) and ten capsid genes ($n=10$) of PCV3 were sequenced. Multiple sequence alignment results showed that these sequences shared 98.5–100% nucleotide similarity with the reference genome sequence and 97.5–100% nucleotide similarity with the reference capsid gene sequence. PCV3 was classified into two different genotypes, according to phylogenetic analysis based on the whole genome. These strains were clustered in PCV3a, showing a close relationship with PCV3-US/SD2016. Surprisingly, we separately analyzed these PCV3 strains from the Guangxi province and found that the dog and pig PCV3 are from different branches. In summary, this was the first seroprevalence and genetic investigation of PCV3 in dogs in the Guangxi province, China, and the first complete genome PCV3 from dogs obtained in the world. The results provide insights into the epidemiology and pathogenesis of this important virus.

1. Introduction

Circoviruses (family *Circoviridae*, genus *Circovirus*) are non-enveloped, circular, single-stranded DNA viruses and can infect various animals (including *Homo sapiens*, pigs, sheep, ducks, and dogs) (Li et al., 2010, 2011). Currently, the genus *Circovirus* includes 11 recognized species (Li et al., 2010).

Canine parvovirus type 2 (CPV-2) is a single-stranded, negative-sense, nonsegmented DNA virus with a genome length of 5.2 kb, belonging to the genus *Parvovirus* of the family *Parvoviridae* (Cotmore et al., 2014). CPV-2 is a common etiological agent that causes severe

gastroenteritis in puppies. The virus has a high detection rate in clinical samples, and the most characteristic signs of this illness are diarrhea, emesis, and anorexia (Apaa et al., 2016). Multiple studies have confirmed that CPV-2 coinfects with more than one enteric pathogen, including Canine enteric coronavirus, Canine circovirus (CanineCV), and Canine distemper virus (Costa et al., 2014; Kotsias et al., 2019; Navarro et al., 2017). T. Thaiwong hypothesizes that the CPV-2 infection predisposes dogs to CanineCV infection and ultimately results in more severe disease (Thaiwong et al., 2016).

Porcine circovirus (PCV) is the smallest autonomously replicating virus with a genome length of 1.7 kb (Cao et al., 2018; Lv et al., 2014).

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The PCV genome contains two major open reading frames (ORFs). ORF1 encodes the replication-associated protein (Rep), which plays an important role in virus replication (Lekcharoensuk et al., 2004; Walia et al., 2014). ORF2 encodes the sole structural protein (capsid protein, Cap), which contains immunologically important epitopes associated with the virus neutralization (Cheung, 2012). Porcine circovirus type 2 (PCV2) infection causes a diverse range of diseases resulting in substantial economic losses to the porcine industry (Segales et al., 2005). It is worth noting that PCV2 could transmit to nonporcine hosts through cross-species transmission routes. Specifically, cattle, dogs and sheep can be infected with PCV2 (Li et al., 2011; Song et al., 2019; Wang et al., 2018; Zhai et al., 2017). Recently, a novel and genetically divergent circovirus, PCV3, was found in swine with PDNS, PMWS in the United States (Palinski et al., 2017). Subsequently, this virus has been detected in many countries and has an abnormally high positive rate (Costa et al., 2014; Deim et al., 2019; Faccini et al., 2017; Saraiva et al., 2019). In China, multiple epidemiological surveillance data indicated that this virus was extensively prevalent in pigs in many provinces and specific cities (Qi et al., 2019; Sun et al., 2018). Multiple studies have revealed that the virus was associated with several diseases (such as PDNS, multisystemic inflammation, reproductive failure) and coinfects with other viruses (for example, Torque teno sus virus, PCV2, porcine reproductive and respiratory syndrome virus) (Chen et al., 2019; Ha et al., 2018; Wen et al., 2018). Retrospective survey studies indicated that PCV3 infection could be traced back to 1996 (Sun et al., 2018). Recent studies confirmed that a PDNS-like disease is reproduced with PCV3 infection alone, and further research suggested that PCV3 is more pathogenic for piglets than PCV2 (Jiang et al., 2019).

Overall, these studies indicated that PCV3 is an important pathogen for pigs and is more pathogenic for piglets than PCV2. Surprisingly, recent studies indicated that dogs could be infected with PCV3 (Zhang et al., 2018). Our previous research also indicated that cattle could be infected with PCV3 (Wang et al., 2019). These results indicate that PCV3 is similar to PCV2 and could transmit to nonporcine hosts, possibly through cross-species transmission routes. However, knowledge about the infection rate and pathogenicity of this virus in dogs is limited. Therefore, we are very interested in investigating the presence of PCV3 in dogs in the Guangxi province, China.

2. Materials and methods

2.1. Sample collection

From 2015–2017, serum samples (n = 406) of dogs were collected from eight cities in the Guangxi, China (Including Nanning, Beihai, Guigang, Liuzhou, Yulin, Hechi, Baise and Guilin) (Fig. 1). This study received animal ethical approval (No. Xidakezi2000138) from Guangxi University (see Ethics approval and consent to participate).

2.2. DNA extraction and polymerase chain reaction (PCR)

The viral genome was extracted from canine serum using the EasyPure Viral DNA/RNA Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Two primer pairs were designed based on the reference sequences of the PCV3/CN/Liaoning-23/2016 strain (NO. KX354055.1) and used published primers and protocols to detect CPV-2 (Table S1). The PCR mixture contained 2 µL of extracted DNA, 2 µL of primer pairs (10 µM), 25 µL of 2×Phanta Max Master Mix (Vazyme, Nanjing, China), and 21 µL of DNase/RNase-Free water. The PCR amplification conditions were as follows: pre-denaturation for 3 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 15 s at 62 °C, an extension for 1 min at 72 °C, then the final extension for 5 min at 72 °C. Subsequently, the PCR products were separated using 1.2% agarose gel electrophoresis of DNA and cloned into a pMD18-T vector (Takara Co. Dalian). The recombinant vectors were amplified in *Escherichia coli* (*E. Coli*, DH5α) for sequencing.

2.3. Multiple sequence alignment and Phylogenetic analysis

The ORF2 gene and genome sequences of PCV3 obtained in this study have been deposited in the GenBank under the accession numbers MH900457–MH900466 and MH916635–MH916638, respectively. In addition, a complete sequence (Guangxi-CZ/05, MH916639) obtained in our previous study was also analyzed in this study. The reference sequences for PCV3 was obtained from the GenBank. Multiple sequence alignments were carried out using Lasergene Package (DNA-STAR Inc.) within the Megalign program (DNASstar software), and the phylogenetic relationships were assessed with the MEGA software (version 7). Support for the phylogenetic relationships was determined by bootstrapping 1000 replicates. In the present study, the method described by Li et al. was used to divide the clades of PCV3 (Li et al., 2018).

3. Results

3.1. Screening for PCV3 prevalence in clinical samples

In this study, we investigated serum samples (n = 406) of dogs. The result indicated that PCV3 was detected in all the cities, 96 out of 406 (23.6%) samples were PCV3-positive samples, 115 of 406 (28.3%) samples were CPV-2 positive samples, 54 of 406 (13.3%) samples were coinfecting with PCV3 and CPV-2 (Table 1). Interestingly, the positive rate of PCV3 ranged from 10.8 to 38.2%, with the highest rate recorded in Baise and the lowest in Yulin (Fig. 1). On the other hand, the highest positive rate of PCV3 ranged from 21.1 to 27.3%, with highest rate recorded in 2017 and the lowest in 2016 (Table S2). Then, the complete genome (n = 4) and the capsid gene (n = 12) were sequenced. Four complete sequences of PCV3 (Guangxi-BH/01, NN/02, LZ/03, GL/04) with a genome length of 2 kb, were similar to most of the reference sequences.

3.2. Multiple sequence alignment and analysis

The multiple sequence alignment of these sequences showed 97.5–100% similarity with the reference capsid gene sequence and 98.5–100% similarity to the reference genome sequence, respectively. The comparison of the complete genome sequences revealed that these strains studied shared 99.7%–100% similarity with each other. In addition, all dog-origin PCV3 strains showed that they shared 98.3% to 100% nucleotide similarity with the ORF2 gene. Similar to previous reports, there are six variant sequences of amino acids 24 to 27 of the Cap (VRRR, VRRK, ARRK, ARRR, ARKR and LRRK), the strains in this study were Cap (24th VRRK 27th). In addition, a comparison of the amino acid sequences revealed a mutation from D124 to N124 in the Cap of Guangxi-C2 and Guangxi-C19 isolate, and a mutation from D124 to N124 in the Cap of Guangxi-NN/02 isolate. Notably, we detected two variant sequences of amino acids 122 of the Rep (V and A), these strains in this study were Rep (A 122th) (Fig. S1).

3.3. Phylogenetic analysis

We used different methods (NJ, ML, and MCC) to reconstruct the phylogenies of the complete PCV3 genome, and different trees displayed similar stably structures in the division of PCV3 into different clades. The phylogenetic divergence analysis based on the complete genome sequences revealed that all the viruses are separated into two major genotypes (PCV3a and PCV3b), and these strains in the present study (Guangxi-BH/01, Guangxi-NN/02, Guangxi-LZ/03, Guangxi-GL/04 and Guangxi-CZ/05) were clustered in a branch representing PCV3a (Fig. 2). The dog-origin PCV3 had a closer relationship with the PCV3-US/SD-2016 strain, PCV3/CN/Chongqing-148/2016 strain, PCV3/CN/Jiangxi-62/2016 strain, and PCV3-BR/RS/8. In contrast, we separately analyzed these strains from Guangxi Province. The dog and pig PCV3 were from different branches.

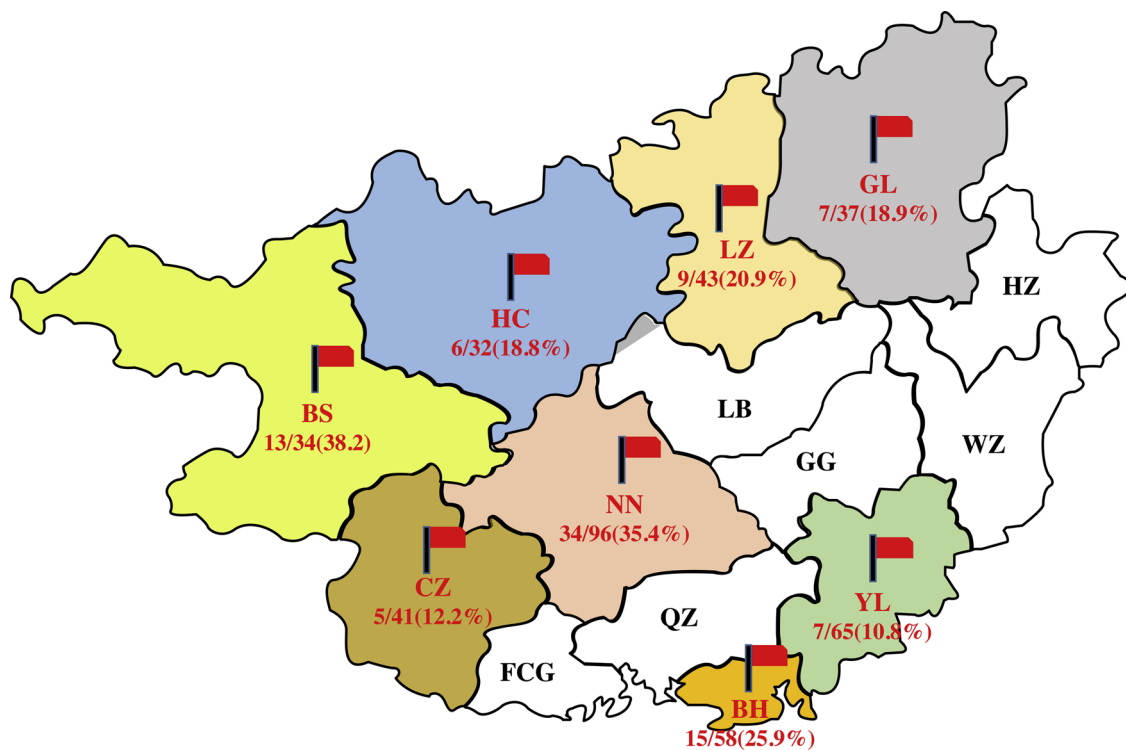


Fig. 1. Geographical distribution of porcine circovirus type 3 (PCV3) in Guangxi Province. The red flag represents the source of the sample and percentage represents positive rate. From which, Guilin, GL; Hezhou, HZ; Liuzhou, LZ; Laibin, LB; Wuzhou, WZ; Guigang, GG; Yulin, YL; Hechi, HC; Nanning, NN; Qinzhou, QZ; Beihai, BH; Baise, BS; Chongzuo, CZ; Fangchenggang, FCG.

Table 1
Frequency and distribution of CPV and PCV3 detected by PCR in samples from 8 cities in Guangxi.

Prefecture	Numbers of samples tested	The positive rate of CPV	The positive rate of PCV3	The positive rate of both CPV and PCV3
Chongzuo	41	9	5	2
Guilin	37	13	7	2
Yulin	65	11	7	3
Nanning	96	32	34	17
Beihai	58	13	15	5
Hechi	32	11	6	2
Liuzhou	43	15	9	3
Baise	34	11	13	4
Total (%)	406	115(28.3%)	96(23.6%)	38(9.4%)

4. Discussion

CPV-2 is a common etiological agent that causes severe gastroenteritis in puppies and coinfects with more than one enteric pathogen (Zhou et al., 2016). T. Thaiwong hypothesizes that CPV-2 infection predisposed dogs to CaCV-1 infection and ultimately resulted in more severe diseases (Thaiwong et al., 2016). Multiple studies indicated that PCV3 is associated with multiple diseases in infected pigs (Jiang et al., 2019). Similar to PCV2, PCV3 also co-infects with other swine pathogens (such as PCV2, classical swine fever virus, PRRSV) (Chen et al., 2019; Zheng et al., 2018). Recent study indicated that dogs could be infected by PCV3 (Zhang et al., 2018), which was found in pig. The results indicated that PCV3 could transmit to non-porcine hosts, possibly through cross-species transmission routes. In this study, the molecular epidemiological investigation results confirmed that coinfection of PCV3 and CPV-2 was prevalent in dogs in the Guangxi province, China.

Multiple studies have confirmed that PCV3 is commonly co-infected with other pathogens (Chen et al., 2019; Ha et al., 2018), but the role of

PCV3 in co-infection remains unclear. In the present study, nearly a quarter (23.6%) of clinical samples were PCV3-positive, and nearly one third (28.3%) of clinical samples were CPV-2-positive. Among them, 38 of 406 (9.36%) samples were coinfecting with PCV3 and CPV-2. Interestingly, the CPV-positive rate was significantly higher in the PCV3-positive samples than non-PCV3 samples, and the difference was extremely significant ($P < 0.01$). Coinfection of pigs with PCV2 and other virus is known to exacerbate the disease severity (Segales et al., 2005; Yao et al., 2019). We identified the coinfection of PCV3 with CPV-2 in dogs and a possible synergy between PCV3 and CPV-2 coinfection with disease severity warrants further investigations.

Previous studies on PCV2 have shown that the amino acid mutation of Rep protein may affect the replication ability of the virus (Cheung, 2012), and the amino acid mutation of Cap protein may change the antigenicity of the virus (Lekcharoensuk et al., 2004). Consistent with previous reports, there are six variant sequences of amino acids 24 to 27 of the Cap (Sun et al., 2018). Several studies suggest that these four amino acids are associated with genetic variation and evolution of PCV3 (Ha et al., 2018; Qi et al., 2019). In addition, we also found mutations in other amino acids of the Cap (D124 to N124 and G160 to R160). However, the mutations in the 124th and 160th amino acid sequences may be unintentional, and these mutations are scattered variations rather than regular variation caused by co-infection (Table S3). Other researches also found co-infection between PCV3 and other pathogens. Unfortunately, they also did not find a relationship between PCV3 genetics and co-infection (Sun et al., 2018).

Currently, there is controversy about the classification of PCV3. In general, most scholars have divided PCV3 into two genotypes (Ha et al., 2018; Li et al., 2018), but there is no consensus on the best classification method. In the present study, we referred to the method described by Li et al, which also divided PCV3 into two different genotypes, PCV3a and PCV3b. Then, by combining genetic evolution analysis with amino acid sequence analysis, it was found that the Rep (A^{122th}) and Cap (24th VRRK 27th or 24th LRRR 27th or 24th VRRR 27th) were mainly in the PCV3a,

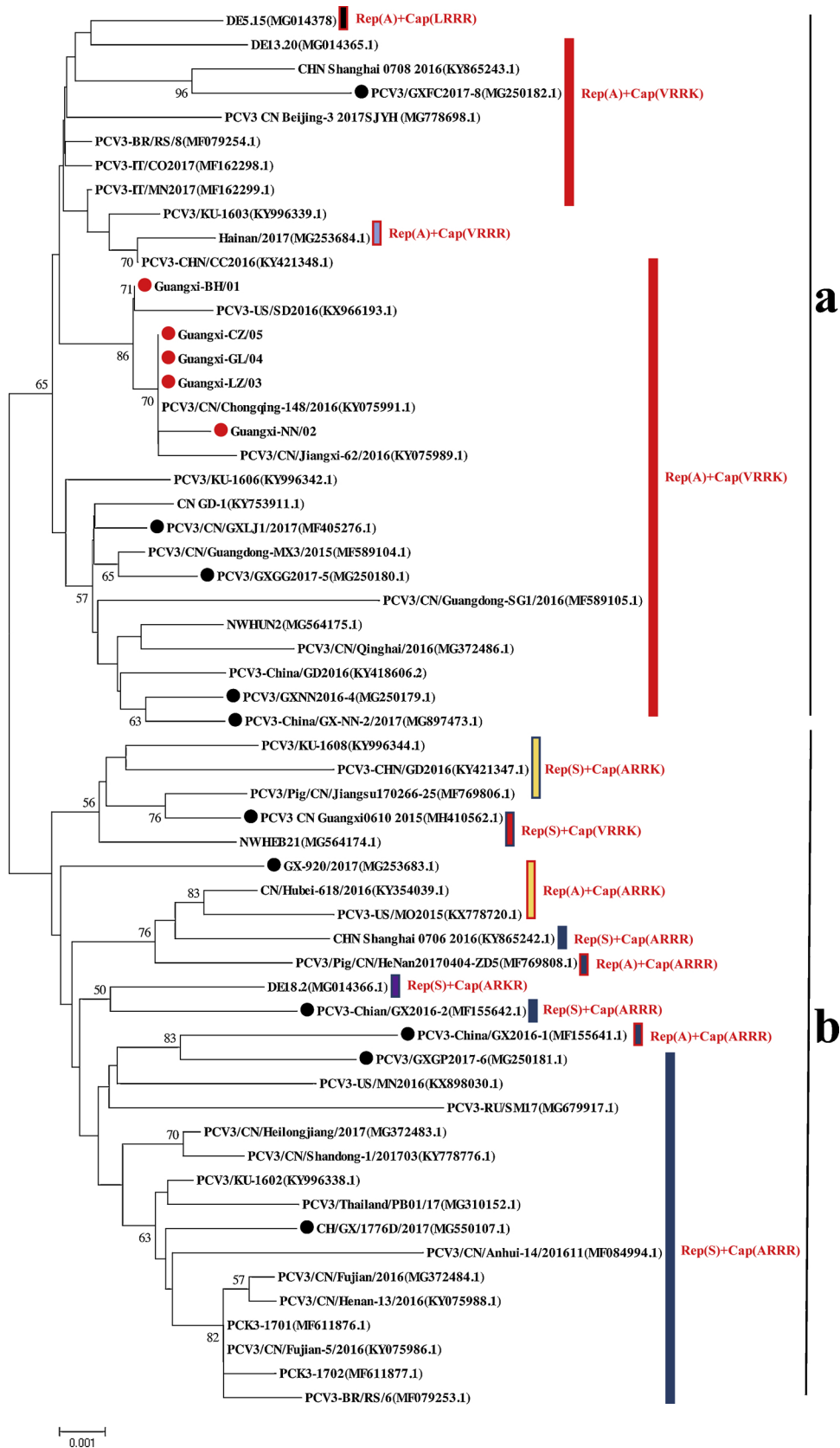


Fig. 2. Phylogenetic analyses of complete genome sequences from PCV3. The Maximum Likelihood (ML) trees were built using 1000 bootstraps replicates. Red circles indicate the strains detected in this study and black circles indicate strains isolated from Guangxi, China. In addition, a complete sequence (Guangxi-CZ/05, MH916639) obtained in our previous study was also analyzed in this study.

and other strains were mainly in the PCV3b. Moreover, the tree displayed stably structures. Therefore, we suggested using the complete genome for PCV3 genotyping and divided PCV3 into a and b subtypes based on the aa codons in Rep (122 aa) and Cap (24aa to 27aa). Notably, all strains in this study were Cap (24th VRRK 27th) and were Rep (A 122th). This result indicates that there is no significant genetic difference in the PCV3 strains obtained in different regions in this study (Table S4). On the other hand, other studies have confirmed that there is no significant genetic difference in PCV3 strains in different regions.

Previous studies have shown that the high genetic stability of pig-origin PCV3 over the past 20 years (Sukmak et al., 2019; Sun et al., 2018). In the present study, the dog-origin PCV3 strain in this study share high nucleic acid similarity (> 95%) with reference sequences of pig-origin PCV3. In addition, compared with pig-origin PCV3 strain, there was no significant amino acid mutation in dog-origin PCV3 strain. Above all, the results of this study indicate that the high genetic stability of dog-origin PCV3. Surprisingly, we separately analyzed these PCV3 strains from the Guangxi Province and found that the dog and pig PCV3 are from different branches. Opriessnig proved that PCV2 in muscle and bone marrow was infectious and transmissible to pigs through the oral route (Opriessnig et al., 2007). The result indicated that PCV3 could transmit to nonporcine hosts by cross-species transmission routes. However, no evidence to prove that PCV3 in muscle and blood of pig was infectious and transmissible to dogs through the oral route. One guess is that PCV3 has been lurking in dogs for a long time.

Declaration of Competing Interest

The authors have no conflict of interest in regard to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.virusres.2019.197663>.

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