Ciência Rural, Santa Maria, v.50:5, e20190909, 2020 http://dx.doi.org/10.1590/0103-8478cr20190909
ISSNe 1678-4596
MICROBIOLOGY

Genomic characterization of *Canine circovirus* detected in a dog with intermittent hemorrhagic gastroenteritis in Brazil

Taís Fukuta Cruz[1,2*] Thiago Neves Batista[3] Ester Mariane Vieira[3] Luiz Marcos Frediani Portela[1,2] Amanda Mahnke Baccarin[3] Jéssica Jehle Gradiz[3] João Pessoa Araújo Junior[1,2]

[1]Departamento de Microbiologia e Imunologia, Instituto de Biociências (IB), Universidade Estadual Paulista (Unesp), 18618-970, Botucatu, São Paulo, Brasil.
[2]Instituto de Biotecnologia (IBTEC), Universidade Estadual Paulista (Unesp), 18607-440, Botucatu, São Paulo, Brasil.
[3]Departamento de Medicina Veterinária, Universidade Regional de Blumenau (FURB), Blumenau, Santa Catarina, Brasil. E-mail: tfcruz@yahoo.com.br.
*Corresponding author.

ABSTRACT: *Because Canine circovirus (CanineCV) is a new species of the genus Circovirus, several issues related to its epidemiology, pathogenesis and clinical disease remain unknown. Thus, this study aimed to perform the characterization of the first complete genome sequence of CanineCV detected in a dog with diarrhea in Brazil. A stool sample was collected of a ten-month-old female German Shepherd dog which had signs of intermittent hemorrhagic gastroenteritis, vomiting, and a history of eating raw pork. The complete CanineCV genome was sequenced by Next-Generation Sequencing. The sequence had 2,063 nucleotides, showed a typical genomic organization for circovirus, and was grouped with strain 214 described in the United States by phylogenetic analysis. One amino acid change was found in the replicase protein, and because of that it was considered unique to CanineCV. Therefore, the characterization of the complete genome of Brazilian CanineCV can be used in future studies of molecular epidemiology, pathogenesis and development of diagnostic tools for the prevention and control of this disease.*
Key words: *circovirus, canine circovirus, genomic characterization, gastroenteritis.*

Caracterização genômica do *Canine circovirus* detectado em um cão com gastroenterite hemorrágica intermitente no Brasil

RESUMO: *Como o Canine circovirus (CanineCV) é uma nova espécie do Gênero Circovirus, várias questões relacionadas com a sua epidemiologia, patogenia e doença clínica permanecem desconhecidas. Assim, este estudo objetivou realizar a caracterização da primeira sequência do genoma completo do CanineCV detectado em um cão com diarreia, no Brasil. Uma amostra de fezes foi coletada de um cão da raça Pastor Alemão, fêmea, 10 meses de idade, o qual tinha sinais de gastroenterite hemorrágica intermitente, vômito e uma história de ingestão de carne crua de porco. O genoma completo do CanineCV foi sequenciado pelo Sequenciamento de Nova Geração. A sequência tinha 2.063 nucleotídeos, apresentou uma organização genômica típica para um circovírus e foi agrupado com a cepa 214, descrita nos Estados Unidos pela análise filogenética. Uma mudança de aminoácido foi encontrada na proteína de replicação e por causa disso ela foi considerada única para o CanineCV. Portanto, a caracterização do genoma completo do CanineCV brasileiro pode ser utilizada em futuros estudos de epidemiologia molecular, patogenia e no desenvolvimento de ferramentas de diagnóstico para prevenção e controle desta doença.*
Palavras-chave: *circovirus, canine circovirus, caracterização genômica, gastroenterite.*

INTRODUCTION

Circoviruses are non-enveloped, icosahedral viruses with single-stranded circular genomic DNA (~ 2 kb) that belong to the family *Circoviridae*. In 2012, a novel species of circovirus (*Canine circovirus*, CanineCV) was first reported in the United States from sera of asymptomatic dogs (KAPOOR et al., 2012). A variant of CanineCV, fox circovirus, was described in foxes with meningoencephalitis in the United Kingdom (BEXTON et al., 2015) and in red foxes in Croatia (LOJKIĆ et al., 2016). In addition, CanineCV has already been detected in wolves, foxes, and badgers in Italy (ZACCARIA et al., 2016). As a new virus, CanineCV has many issues related to its pathogenesis and clinical disease development that need to be elucidated because it has been associated with cases of hemorrhage, vasculitis, granulomatous lymphadenitis, severe gastroenteritis, and respiratory signs (LI et al., 2013; DECARO et al., 2014; HSU et al., 2016; THAIWONG et al., 2016; ZACCARIA et al., 2016; PIEWBANG et al., 2018). Furthermore, it has been suggested that cases

Received 11.16.19 Approved 02.21.20 Returned by the author 04.01.20
CR-2019-0909.R2

of CanineCV coinfections may lead to more severe clinical illnesses (LI et al., 2013; THAIWONG et al., 2016; ANDERSON et al., 2017; DOWGIER et al., 2017), which is similar to what occurs with porcine circovirus 2 (PCV2) (OPRIESSNIG & HALBUR, 2012). So far, CanineCV has been reported in countries in North America (KAPOOR et al., 2012; LI et al., 2013; THAIWONG et al., 2016), South America (WEBER et al., 2018; KOTSIAS et al., 2019), Europe (DECARO et al., 2014; ZACCARIA et al., 2016; ANDERSON et al., 2017; DOWGIER et al., 2017), and Asia (HSU et al., 2016; PIEWBANG et al., 2018; SUN et al., 2019). Thus, the objective of this study was to perform the genomic characterization of CanineCV detected in a dog with intermittent hemorrhagic gastroenteritis in Brazil.

MATERIALS AND METHODS

This study was approved by the Committee on Ethics in the Use of Animals (CEUA) from Regional University of Blumenau (FURB) under protocol nº 006/13. In January 2013, a female ten-month-old German Shepherd dog, un-spayed, vaccinated against giardiasis, canine adenovirus type 1 and 2, canine distemper virus, canine parvovirus, parainfluenza virus, coronavirus, and *Leptospira* spp., presented intermittent hemorrhagic gastroenteritis associated with melena, some emetic episodes, 38.9 °C body temperature, and absence of abdominal pain. In addition, the dog was constantly fed with raw pork. Initially, giardiasis was suspected, but a fecal examination performed by zinc sulphate flotation and salt flotation techniques was negative for helminth eggs and protozoan oocysts; nevertheless, symptomatic treatment was conducted with fluid therapy and antibiotics (metronidazole) for giardiasis, with no adequate resolution of the clinical signs. The infection time course was three months with intermittent diarrhea, and there was no evidence of transmission to other dogs. Therefore, to establish an etiological diagnosis, a stool sample was molecularly analyzed for the presence of *Cryptosporidium* spp., *Giardia duodenalis*, canine parvovirus (CPV), CanineCV, and porcine circovirus (PCV) DNA.

The stool sample (0.2 g) was subjected to a freezing cycle (-196 °C/2 min) and thawing (60 °C/2 min). Then, the DNA was extracted using a QIAamp® DNA Stool Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

PCR and nested-PCR were used as described by XIAO et al. (2001) for the DNA detection of *Cryptosporidium* spp. PCR and semi-nested PCR for *Giardia duodenalis* were also prepared as reported by READ et al. (2004).

Quantitative PCR (qPCR) was performed as described for CPV (DECARO et al., 2005), CanineCV (LI et al., 2013) and PCV (LADEKJAER-MIKKELSEN et al., 2002) with some modifications. This way, qPCRs based on SYBR Green were qualitatively evaluated using melting curve concordance and the values of the cycle threshold (Ct). Each qPCR was prepared separately with a GoTaq® qPCR Master Mix kit (Promega, Madison, WI), following the manufacturer's recommendations. The primer pairs used in each qPCR had concentrations 0.4 µM (CPV-qPCR), 0.3 µM (CanineCV-qPCR), and 0.2 µM (PCV-qPCR). In CPV-qPCR, the thermocycling conditions were 95 °C/2 min and 40 cycles of 95 °C/15 sec, 52 ºC/30 sec, and 60°C/1 min, followed by a melting curve from 95°C to 60 °C. For CanineCV-qPCR and PCV-qPCR, the reaction conditions were 95 ºC/2 min and 40 cycles of 95 ºC/15 sec and 60ºC/1 min, followed by a melting curve from 95 °C to 60 °C. The qPCRs were performed in a 7300 Real Time PCR System (Life Technologies, Foster City, CA).

The complete genome of CanineCV was amplified by rolling circle amplification (RCA) using the enzyme phi29 DNA polymerase (Thermo Fisher Scientific, Carlsbad, CA), following the manufacturer's recommendations. The digestion reaction with the enzyme NcoI (New England Biolabs, Ipswich, England) was performed to confirm the presence of expected product size of the complete CanineCV genome, according to the manufacturer's instructions. After visualizing of 1% agarose gel stained with Brilliant Green Plus (Neotaq, Botucatu, Brazil) by UV light exposure (Figure 1), DNA band of expected size was purified with a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, United Kingdom) and quantified by Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Carlsbad, CA), following the manufacturer's instructions.

The sequencing library was prepared using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA), and the sequencing was performed on the Illumina NextSeq platform (2 x 150 cycles) (Illumina, San Diego, CA) following the manufacturer's recommendations. The reads generated were analyzed and assembled with Geneious v.6.1.8 (KEARSE et al., 2012) to obtain the complete CanineCV D1056 genome. The complete genome sequence, Open Reading Frame 1 (ORF1) and Open Reading Frame 2 (ORF2) of CanineCV D1056 were aligned with 48 CanineCV sequences

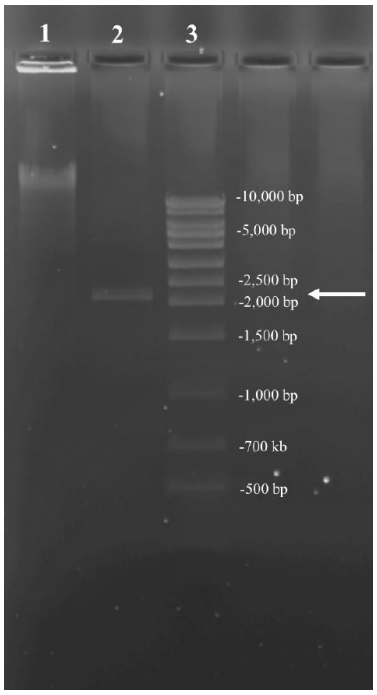

Figure 1 - Agarose gel (1%) electrophoresis of CanineCV after rolling circle amplification (RCA) and digestion reaction with enzyme NcoI. Lane 1: product of RCA. Lane 2: product of digestion reaction. Lane 3: High Ranger 1 kb DNA Ladder (Norgen). The expected size (~ 2 kb) of the complete CanineCV genome is indicated by the arrow.

deposited on GenBank using ClustalW (LARKIN et al., 2007) implemented in MEGA v.6 (TAMURA et al., 2013). Afterwards, a phylogenetic tree was constructed based on the nucleotide sequence for the complete CanineCV D1056 genome by the Maximum Likelihood method (MEGA v.6) using the GTR+G as a substitution model and 1,000 bootstrap replicates. The complete CanineCV D1056 genome was deposited in GenBank under accession number MK424788.

The attempt to isolate CanineCV D1056 was performed in MDCK (Madin-Darby canine kidney) cell culture according to the procedure described for PCV2 (CRUZ & ARAUJO JR, 2014), with some modifications. A 6.25% stool suspension was prepared with minimum essential media (MEM) (Gibco, Grand Island, NE) and 0.5X Antibiotic-Antimycotic 100X (Gibco, Grand Island, NE). The suspension was centrifuged at 3,220 x g for 15 min at 4 ºC, and the supernatant was filtered through a 0.22 µm sterile membrane. The inoculation was performed after the trypsinization of the control and infected MDCK cells. Five passages were performed. Between each subculture, the cells were treated with 100 mM D-glucosamine pH 7.0. Aliquots of the control and infected cell suspensions of each passage were separated for DNA extraction using an Illustra Blood GenomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. Finally, qPCR for CanineCV was performed as reported above.

RESULTS AND DISCUSSION

In this study, the stool sample was positive for CanineCV (Ct value 32) by qPCR. Analysis of the

complete CanineCV D1056 sequence showed that it was 2,063 nt in length, 51% GC, and a genomic organization typical of the circovirus, as previously reported for CanineCV (KAPOOR et al., 2012; LI et al., 2013). Thus, two major ORFs, 1 and 2 in opposite orientation, were reported in CanineCV D1056 genome, which encode the viral replicase (Rep, 303 amino acids, aa) and capsid protein (Cap, 270 aa), respectively. In addition, two noncoding intergenic regions were reported in the viral genome. The 5'-intergenic region (135 nt in length) between the start codons of the two major ORFs contains a typical stem-loop structure for the initiation of rolling-circle replication and the conserved 9-nt sequence (TAGTATTAC) between the circoviruses. The 3'-intergenic region (203 nt in length) is located between the stop codons of the two major ORFs. Sequences of amino acids highly conserved among circoviruses associated with rolling-circle replication (FTINN, TPHLQ and CSK) and the dNTP binding (GCGKS) were identified in the replicase protein. For the capsid protein, the N-terminus region was rich in arginine residues, as usually observed in circoviruses.

The nucleotide identity shared among CanineCV D1056 with the other 48 CanineCV sequences for complete genome, replicase and capsid nucleotide sequences was 83.5% - 98.0%, 81.1% - 98.5% and 80.3% - 98.3%, respectively. The phylogenetic tree showed that CanineCV D1056 was grouped with strain 214 (GenBank JQ821392.1) (Figure 2). Strain 214 was described in the United States in 2012 from asymptomatic dog sera and

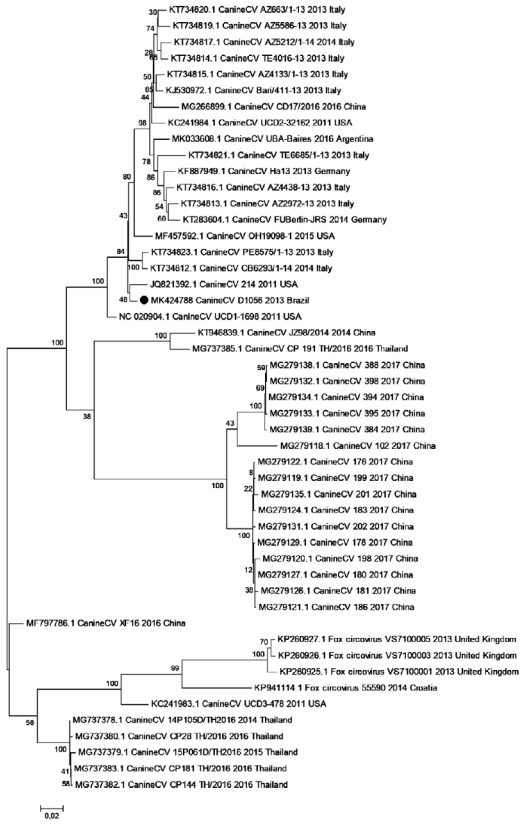

Figure 2 - Phylogenetic tree based on the complete nucleotide sequence of the CanineCV D1056. The tree was constructed with the Maximum Likelihood method (MEGA v.6) using the GTR+G as substitution model. The bootstrap of 1,000 replicates was used in the tree. The analysis involved 48 nucleotide sequences deposited in GenBank. The complete genome of CanineCV D1056 (GenBank MK424788) is highlighted as a black circle.

was the first complete CanineCV sequence reported (KAPOOR et al., 2012). The nucleotide identities found for the two sequences with respect to the complete genome, replicase and capsid were 98%, 97.3% and 98.3%, respectively. For amino acid identities, the values found were 96.7% and 98.9% for replicase and capsid proteins, respectively. In the protein residue 16 of the replicase protein, one amino acid change was considered unique to CanineCV D1056 (Cysteine) compared with other CanineCV (Glycine) sequences used in this study. In Brazil, a CanineCV contig of 200 bases in length was obtained after the dog virome characterization in 2018 (WEBER et al., 2018). However, the authors did not determine the complete CanineCV genome, and there was no description of the clinical signs associated with this sequence. Consequently, the first complete CanineCV genome sequencing in Brazil associated with intermittent hemorrhagic gastroenteritis in a dog in January 2013 was reported in this study.

The animal was negative for *Cryptosporidium* spp., *Giardia duodenalis*, and CPV. For CanineCV, it is suggested that the occurrence of coinfections may increase the severity of the clinical disease (LI et al., 2013; THAIWONG et al., 2016; ANDERSON et al., 2017; DOWGIER et al., 2017). Moreover, a higher frequency of CanineCV infections had been previously described in younger animals (< 1 year old) (HSU et al., 2016; ZACCARIA et al., 2016; DOWGIER et al., 2017). Such characteristic was also observed in the present case since the dog was ten months old.

As formerly described by the frequent intake of raw pork by the animal, qPCR for PCV was performed and resulted negative. However, since domestic dog infection with PCV2a (HERBST & WILLEMS, 2017) and PCV3 (ZHANG et al., 2018) has already been reported, it is important not to provide domestic dogs with raw meat because of the high risk of transmission of various pathogens, including zoonotic agents.

The attempt to isolate CanineCV D1056 in MDCK cells was unsuccessful, since viral DNA was detected by qPCR only during the inoculation phase. As the amount of viral DNA found by qPCR for CanineCV was low, this may have hindered viral isolation. Moreover, the presence of viral DNA in the inoculum does not indicate the existence of an integral viral particle, which is necessary for the virus to adsorb in the cells.

CONCLUSION

It is known that the pathogenesis and development of clinical diseases caused by CanineCV remain uncertain, so several studies are essential to obtain a better understanding of the factors involved in the interaction of this viral agent with its hosts. Hence, the present study was a pioneer in performing the genetic characterization of Brazilian CanineCV D1056 detected in a female dog with intermittent hemorrhagic gastroenteritis, which can assist in future research related to the molecular epidemiology, pathogenesis, and development of serological and molecular diagnostic methods for CanineCV detection.

ACKNOWLEDGEMENTS

This study was supported by the São Paulo Research Foundation (FAPESP) (grant number 2016/09518-0), Institute of Biosciences Foundation (grant number 011/2015), and was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasil – Finance code 001.

DECLARATION OF CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

All procedures performed in this study involving animals were in accordance with the ethical standards of the Committee on Ethics in the Use of Animals (CEUA) from Regional University of Blumenau (FURB) under protocol nº 006/13.

AUTHORS' CONTRIBUTIONS

TFC, TNB, and JPAJ: conceived the experiments. TFC, TNB, EMV, LMFP, AMB, and JJG: performed experiments and analyzed data. TFC: wrote the paper. All authors read, edited, and approved the final manuscript.

REFERENCES

ANDERSON, A. et al. Role of canine circovirus in dogs with acute haemorrhagic diarrhoea. **The Veterinary Record**, v. 180, n.22, p.542, 2017. Available from: <https://veterinaryrecord.bmj.com/content/180/22/542.long>. Accessed: Mar. 20, 2018. doi: 10.1136/vr.103926.

BEXTON, S. et al. Detection of circovirus in foxes with meningoencephalitis, United Kingdom, 2009-2013. **Emerging Infectious Diseases**, v.21, n.7, p.1205-1208, 2015. Available from: <https://wwwnc.cdc.gov/eid/article/21/7/15-0228_article>. Accessed Mar. 20, 2018. doi: 10.3201/eid2107.150228.

CRUZ, T.F.; ARAUJO JR, J.P. Cultivation of PCV2 in swine testicle cells using the shell vial technique and monitoring of viral replication

by qPCR and RT-qPCR. **Journal of Virological Methods**, v.196, p.82-85, 2014. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S0166093413004291?via%3Dihub>. Accessed: Mar. 20, 2018. doi: 10.1016/j.jviromet.2013.10.025.

DECARO, N. et al. A real-time PCR assay for rapid detection and quantitation of canine parvovirus type 2 in the feces of dogs. **Veterinary Microbiology**, v.105, n.1, p:19-28, 2005. Available from: <https://www.sciencedirect.com/science/article/pii/S0378113504003554?via%3Dihub>. Accessed: Mar. 20, 2018. doi: 10.1016/j.vetmic.2004.09.018.

DECARO, N. et al. Genomic characterization of a circovirus associated with fatal hemorrhagic enteritis in dog, Italy. **PLoS One**, v.9, n.8, e105909, 2014. Available from: <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0105909>. Accessed: Mar. 20, 2018. doi: 10.1371/journal.pone.0105909.

DOWGIER, G. et al. A molecular survey for selected viral enteropathogens revealed a limited role of Canine circovirus in the development of canine acute gastroenteritis. **Veterinary Microbiology**, v.204, p.54-58, 2017. Available from: <https://www.sciencedirect.com/science/article/pii/S0378113517302389?via%3Dihub>. Accessed: Mar. 20, 2018. doi: 10.1016/j.vetmic.2017.04.007.

HERBST, W.; WILLEMS, H. Detection of virus particles resembling circovirus and porcine circovirus 2a (PCV2a) sequences in feces of dogs. **Research in Veterinary Science**, v.115, p.51-53, 2017. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S0034528817300693?via%3Dihub>. Accessed: Mar. 20, 2018. doi: 10.1016/j.rvsc.2017.01.014.

HSU, H.S. et al. High detection rate of dog circovirus in diarrheal dogs. **BMC Veterinary Research**, v.12, n.1, p.116, 2016. Available from: <https://bmcvetres.biomedcentral.com/articles/10.1186/s12917-016-0722-8>. Accessed: Mar. 20, 2018. doi: 10.1186/s12917-016-0722-8.

KAPOOR, A. et al. Complete genome sequence of the first canine circovirus. **Journal of Virology**, v.86, n.12, p.7018, 2012. Available from: <https://jvi.asm.org/content/86/12/7018.long>. Accessed: Mar. 20, 2018. doi:10.1128/JVI.00791-12.

KEARSE, M. et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. **Bioinformatics**, v.28, n.12, p.1647-1649, 2012. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3371832/>. Accessed: Mar. 20, 2018. doi: 10.1093/bioinformatics/bts199.

KOTSIAS, F. et al. Genomic characterization of canine circovirus associated with fatal disease in dogs in South America. **PLoS One**, v.14, n.6, e0218735, 2019. Available from: <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0218735>. Accessed: Aug. 10, 2019. doi: 10.1371/journal.pone.0218735.

LADEKJAER-MIKKELSEN, A.S. et al. Reproduction of postweaning multisystemic wasting syndrome (PMWS) in immunostimulated and non-immunostimulated 3-week-old piglets experimentally infected with porcine circovirus type 2 (PCV2). **Veterinary Microbiology**, v.89, n.2-3, p.97-114, 2002. Available from: <https://www.sciencedirect.com/science/article/pii/S0378113502001748?via%3Dihub>. Accessed: Apr. 23, 2004. doi: 10.1016/s0378-1135(02)00174-8.

LARKIN, M.A. et al. Clustal W and Clustal X version 2.0. **Bioinformatics**, v.23, n.21, p.2947-2948, 2007. Available from: <https://academic.oup.com/bioinformatics/article/23/21/2947/371686>. Accessed: Jun. 17, 2010. doi: 10.1093/bioinformatics/btm404.

LI, L. et al. Circovirus in tissues of dogs with vasculitis and hemorrhage. **Emerging Infectious Diseases**, v.19, n.4, p.534-541, 2013. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3647419/>. Accessed: Aug. 5, 2014. doi: 10.3201/eid1904.121390.

LOJKIĆ, I. et al. Faecal virome of red foxes from peri-urban areas. **Comparative Immunology, Microbiology and Infectious Diseases**, v.45, p.10-15, 2016. Available from: <https://www.sciencedirect.com/science/article/pii/S0147957116300121?via%3Dihub>. Accessed: Mar. 20, 2018. doi: 10.1016/j.cimid.2016.01.005.

OPRIESSNIG, T.; HALBUR, P.G. Concurrent infections are important for expression of porcine circovirus associated disease. **Virus Research**, v.164, n.1-2, p.20-32, 2012. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S0168170211003510?via%3Dihub>. Accessed: Jun. 27, 2013. doi: 10.1016/j.virusres.2011.09.014.

PIEWBANG, C. et al. Novel canine circovirus strains from Thailand: Evidence for genetic recombination. **Scientific Reports**, v.8, n.1, p.7524, 2018. Available from: <https://www.nature.com/articles/s41598-018-25936-1>. Accessed: Mar. 20, 2018. doi: 10.1038/s41598-018-25936-1.

READ, C.M. et al. Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. **Infection, Genetics and Evolution**, v.4, n.2, p.125-130, 2004. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S1567134804000139?via%3Dihub>. Accessed: Feb. 13, 2005. doi.org/10.1016/j.meegid.2004.02.001.

SUN, W. et al. The detection of canine circovirus in Guangxi, China. **Virus Research**, v.259, p.85-89, 2019. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S0168170218303927?via%3Dihub>. Accessed: Oct. 15, 2019. doi: 10.1016/j.virusres.2018.10.021.

TAMURA, K. et al. MEGA6: Molecular evolutionary genetics analysis version 6.0. **Molecular Biology and Evolution**, v.30, n.12, p.2725-2729, 2013. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3840312/>. Accessed: Sept. 17, 2014. doi: 10.1093/molbev/mst197.

THAIWONG, T. et al. Canine circovirus 1 (CaCV-1) and canine parvovirus 2 (CPV-2): recurrent dual infections in a papillon breeding colony. **Veterinary Pathology**, v.53, n.6, p.1204-1209, 2016. Available from: <https://journals.sagepub.com/doi/full/10.1177/0300985816646430?url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub%3Dpubmed>. Accessed: Mar. 20, 2018. doi: 10.1177/0300985816646430.

WEBER, M.N. et al. Characterization of dog serum virome from Northeastern Brazil. **Virology**, v.525, p.192-199, 2018. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S0042682218303015?via%3Dihub>. Accessed: Jan. 27, 2019. doi: 10.1016/j.virol.2018.09.023.

XIAO, L. et al. Molecular characterization of cryptosporidium oocysts in samples of raw surface water and wastewater. **Applied and Environmental Microbiology**, v.67, n.3, p.1097-1101, 2001. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC92700/>. Accessed: Feb. 10, 2005. doi: 10.1128/AEM.67.3.1097-1101.2001.

ZACCARIA, G. et al. Circovirus in domestic and wild carnivores: An important opportunistic agent? **Virology**, v.490, p.69-74, 2016. Available from: <https://www.sciencedirect.com/science/article/pii/S0042682216000106?via%3Dihub>. Accessed: Mar. 20, 2018. doi: 10.1016/j.virol.2016.01.007.

ZHANG, J. et al. First molecular detection of porcine circovirus type 3 in dogs in China. **Virus Genes**, v.54, n.1, p.140-144, 2018. Available from: <https://link.springer.com/article/10.1007%2Fs11262-017-1509-0>. Accessed: Jun. 27, 2018. doi: 10.1007/s11262-017-1509-0.